1975

Helper and Suppressor Functions of Thymus-Derived Lymphocytes in the Humoral Response to Heterologous Erythrocytes in Rabbits.

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HELPER AND SUPPRESSOR FUNCTIONS OF THYMUS-DERIVED LYMPHOCYTES IN THE HUMORAL RESPONSE TO HETEROLOGOUS ERYTHROCYTES IN RABBITS

A DISSERTATION

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Microbiology

by
Roland Ray Arnold
B.S., Louisiana State University, 1972

August, 1975

ACKNOWLEDGEMENT

The author wishes to express his deep appreciation to Dr. R. J. Siebeling for his guidance, understanding and assistance during this investigation and in the preparation of this dissertation. He also wishes to express gratitude to Dr. H. D. Braymer, Dr. A. D. Larson and Dr. J. G. Lee for their many contributions during his graduate years. The author would like to thank Dr. M. D. Socolofsky, Chairman of the Department of Microbiology, and all the other members of the faculty of the Department of Microbiology for their encouragement and understanding.

The author wishes to thank Paul Landry for his assistance and friendship, both of which have been invaluble. Russell Roberts and Tom Moore both made significant technical contributions to this study. Special thanks are extended to Frank Hatcher and other fellow graduate students whose aid and friendship were greatly appreciated.

A very special thanks is extended to his wife, Mona, and his son, Todd, whose thoughtfulness, understanding, patience and sacrifice made this investigation possible.

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GLOSSARY OF ABBREVIATIONS

Ab	Antibody
AFC	Antibody forming cell
Ag	Antigen
AIS	Antigen-induced suppression
ALS	Antilymphocyte serum
ARC	Antigen reactive cell
ATGG	Antithymocyte gamma globulin
ATS	Antithymocyte serum
B-cell	Precursor of AFC
BM	Bone marrow
CI	Cytotoxicity index
HARTS	Horse anti-rabbit thymocyte serum
HBSS	Hank's balanced salt solution
HRBC	Horse red blood cells
im	Intramuscular
ip	Intraperitoneal
iv	Intravenous
NHGG	Normal horse gamma globulin
PFC	Plaque-forming cells
SRBC	Sheep red blood cells
R	Rads
RLN	Regional lymph nodes
T-cell	Thymus-derived lymphocytes
TCM	Tissue culture medium
WBC	White blood cells

ABSTRACT

Two functions, helper and suppressor activity, of the thymus-derived lymphocyte (T-cell) in the humoral antibody response of rabbits to sheep erythrocytes (SRBC) were investigated. In the first aspect of this study splenic plaque-forming cell (PFC) and humoral hemolysin responses to SRBC were examined in adult thymectomized, lethally x-irradiated rabbits infused with allogeneic bone marrow (BM) and thymus cells from cortisone- treated littermate donors. Seven to eight week old rabbits were thymectomized and at eight to ten weeks of age received 900R total body x-irradiation. One portion of this group was infused intravenously (iv) with the total BM (B-cell source) and thymus cell populations from cortisone-treated littermate donors. Another group received 20×10^8 nucleated BM cells. The remaining animals received no reconstituting cell populations. All groups were immunized iv with 1.0 ml of a 10 percent suspension of SRBC. Peripheral white blood cell counts (WBC) and anti-SRBC hemolysin titers were determined on selected days after immunization. Representative animals from each group were killed on the seventh day after immunization and their spleens were assayed for anti-SRBC PFC activity. Hemolysin responses in the BM reconstituted and non-reconstituted animals were minimal when compared to the control group responses. Those animals

which received both BM and thymus cells showed a delayed but significant rise in hemolysin responsiveness which approached that of the normal control group. The PFC data reflected the hemolysin responses as the non-reconstituted and BM reconstituted animals showed responses less than background. The infusion of BM and thymus cells significantly restored the number of PFC (48.3 per million cells) when compared to the normal control response (50.8 per million cells). These data support the need for synergism between the T- and B-cell population in the humoral response to SRBC in rabbits.

In the second aspect of this study horse anti-rabbit thymus gamma globulin (ATGG) was used to investigate the suppressor function of the T-cell in antigenic competition between heterologous erythrocytes. Eight week old rabbits were injected iv with 1.0 ml of a 20% horse red blood cell suspension (2xHRBC) followed three days later with 1xSRBC. ATGG was injected two days before, on the same day and two days after HRBC injection (Test group). The effect of ATGG was controlled by substituting normal horse gamma globulin (NHGG) or ATGG which had been adsorbed with thymocytes (ATGG-adsorbed control). An additional control group was injected with HRBC and SRBC only (competition). Anti-SRBC PFC responses were assayed on the 5th day post SRBC injection. The competition group showed a

supressed response (29 PFC/10^6) when compared to the normal non-competitive anti-SRBC responses (72 PFC/10^6). ATGG-treatment alleviated this antigen-induced suppression and these animals showed a marginally enhanced response (90 PFC/10^6). The NHGG and ATGG-adsorbed control groups were not significantly different from the competition group. The anti-SRBC hemolysin responses reflected the PFC data. These results suggest ATGG interfered with the suppressive effect of a priming injection of HRBC on the subsequent anti-SRBC response in rabbits.

Chapter 1

REVIEW OF LITERATURE

Introduction

One of the major advances in immunobiology was the demonstration that there exists a dichotomy of function for immunocomp tent lymphocytes residing with two distinct lymphoid cell populations. It is generally accepted that both of these populations are derived from BM stem cells which differentiate to immunocompetence under distinct anatomical influences. One cell type, the T-cell, differentiates to maturity under the influence of the thymus and is responsible for the various aspects of cell-mediated-immunity: delayed-type hypersensitivity, homograft, mixed lymphocyte and graft-versus-host reactions. The second lymphocyte type, which also arises from the BM, does not come under thymus influence and populates ultimately specific anatomical sites in the peripheral lymphoid tissue distinct from areas populated by T-cells. This cell, known as the B-cell, is the precursor of the antibody producing plasma cell.

Recent research has revealed that differentiation of the B-cell to that of an immunoglobulin synthesizing plasma cell requires in most instances the simultaneous

antigenic stimulation and differentiation of T- cells in an auxiliary or helper capacity. More recently it has become apparent that in addition to this well established helper function, the T-cell may act under certain circumstances to suppress the immune response and that this suppressor function may be responsible for antigen-induced suppression observed in the phenomenon known as antigenic competition. It now seems that what at first appeared to be simple cooperation between a two cell system (T and B cells) in order to initiate antibody production in response to a specific antigen actually encompasses the expression of a regulatory function that activated T-cells may exert on antigen stimulated B-cells. The following review is intended to present experimental evidence which support the existence of a regulatory role for the T-cell and also to elaborate on the nature and function of the two lymphocyte cell types.

Ontogeny of T and B Lymphocytes

Current evidence suggests that both the T and the B-cell arise from the same pluripotential stem cells (Figure 1) which originate in the embryonic yolk sac and primitive blood islands and subsequently migrate to establish hemopoetic colonies in the fetal liver and bone marrow (Ford et al., 1966; Owen and Ritter, 1969; Miller and Mitchell, 1969). Certain of these precursor cells migrate in turn to the par-

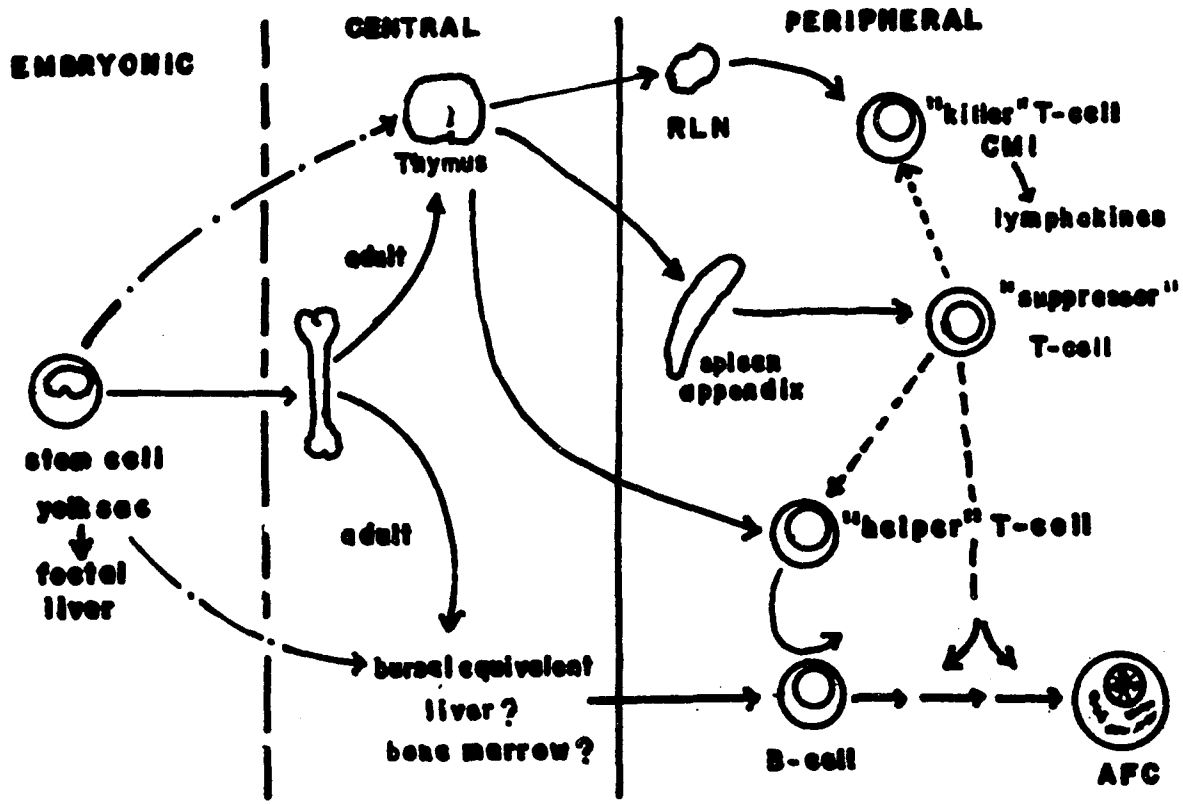

Figure 1. Postulated roles played by the various lymphoid organs on the ontogeny and expression of the different lymphoid cell types.

enchyma of the thymus which at that time consist of reticular epithelial cells derived embryonically from the visceral pouches of the third and fourth pharyngeal clefts (Clark, 1973). The epithelial cell compartment of the thymus reportedly secretes glycoproteins, which have the ability to induce T-cell development and maturation (Clark, 1968 & 1963; White,1975; Goldstein,1975; Waksal et al., 1975). There is also some evidence that thymus influenced differentiation may continue after the T-cells exit this tissue and peripheralize (Cohen et al., 1975).

In the avian system B-cell differentiation of the precursor stem cells has clearly been shown to occur in the bursa of Fabricius, a primary lymphoid structure (Glick et al., 1956; Warner et al., 1962). The tissue site for B-cell differentiation in the mammalian system has not been clearly elucidated, as a discrete organ of bursal equivalence has not been found. However, recent evidence indicates that the liver may, at least in part, be the site for B-cell differentiation (Owen et al., 1974; Cooper et al., 1975). It has also been suggested that the bone marrow is the mammalian analogue of the bursa of Fabricius and therefore the site of B-cell maturation (Claman, 1975).

T-Cell Dependent Antibody Production

It is generally accepted that there are two distinct types of immunological responsiveness to antigen stimulation; cell-mediated-immunity (CMI) and humoral mediated immunity (antibody protein). When mice are neonatally thymectomized (within 24 hours of birth) the T-cell population is effectively eliminated before it has an adequate opportunity to peripheralize (Takahasi et al., 1970; Goldschneider and McGregor, 1966). As a consequence of T-cell depletion, there is a concurrent loss in the ability to express CMI in the mouse (Papermaster et al., 1962; Miller, 1961 & 1962; Martinez et al., 1962) and in the chicken (Glick et al.,1956; Warner et al., 1962). The absent thymocyte function can be restored with thymus grafts implanted in the thymectomized animal (Miller, 1961 & 1962; Dalmasso et al., 1963; Taylor, 1963; Leuchars et al., 1965). Similarly bursectomy shortly before or after hatching will lead to severe impairment of the humoral Ab response, while leaving CMI response capabilities intact (Glick et al., 1956; Warner et al., 1962). Such investigations established that the bursal-derived lymphocyte was responsible for differentiation and maturation of the Ab producing system and the T-cell for cell-mediated immunity.

Miller (1961 & 1962) complicated the picture when he showed that the depletion of T-cells by neonatal thymec-

tomy of mice resulted in a diminished humoral antibody response when these animals were immunized with foreign serum proteins. This deficiency could be restored with thymus allografts. This suggested a role for the thymus-derived lymphocyte in humoral antibody responses. Miller et al. (1964) further implicated the T-cell in the humoral response by demonstrating that irradiated mice could be reconstituted to full immunocompetence with infusions of syngeneic BM, however, only if the recipient's thymus was present. Osoba and Miller (1963) showed that the immunological capacity of neonatally thymectomized mice could be partially restored by implanting thymus tissue enclosed in a Millipore diffusion chamber which prevented cell traffic into or out of the chamber, thus suggesting an endocrine function for the thymus.

Some insight into the nature of the roles of the two cells was implied by the experiments of Kennedy et al. (1965) and Gregory and Lajtha (1968). Lethally irradiated mice received iv infusions of syngeneic lymphoid cells from various sources (ie. spleen, thymus, and BM) and then the recipient spleens were cut into thin serial slices 8 days after SRBC immunization. The spleen sections were assayed for hemolytic foci or clusters of antibody forming cells (Kennedy et al., 1965). Hemolytic foci were apparent only when recipients were reconstituted with spleen or lymph

node cells, but not when either BM or thymocytes were infused alone. The data suggested that each cluster of AFC resulted from the activity of a single antigen reactive cell which had lodged in the recipient spleen, proliferated and differentiated as a result of SRBC antigenic stimulation. The number of foci (cell clusters) increased linearly with the number of spleen cells transferred (slope of 1); whereas, the number of individual PFC increased disproportionately (slope of 2), which implied that the development of a hemolytic focus was dependent upon the presence of one cell, while the AFC maturation or expression required the interaction of 2 cell types (Gregory and Lajtha, 1968).

The first direct evidence for T-and B- cell synergism was reported by Claman et al. (1966). These investigators showed that optimum splenic hemolytic foci (Playfair et al., 1965) responsiveness could be restored in lethally x-irradiated mice by infusing both BM cells and thymocytes, but not when either population was infused alone. The quantity of thymocytes or the BM cells infused was varied and the quantity of the other cell population was kept constant (at optimal functional dose) and it was found that the number of hemolytic foci increased proportionally with the number of thymus cells infused. From this finding it was postulated that the BM cell constituted the source for the

AFC and that this cell population only expressed itself in the presence of an auxiliary cell of thymic origin.

This hypothesis was subsequently substantiated by others. Immunological responsiveness to SRBC was restored when neonatally thymectomized mice received either allogeneic thoracic duct or thymus lymphocytes (Mitchell and Miller, 1968). Thoracic duct cells appeared to be a better source for immunocompetent T-cells than the thymus, since thymocytes must first react with antigen before interaction and/or recruitment of BM cells for the production of anti-SRBC antibody. More significantly it was shown that *in vitro* PFC activity could be inhibited when treated with anti-H-2 sera directed against host, but not donor cells. This latter observation proved the AFC was BM derived and the ARC was thymus derived (Miller and Mitchell, 1968). The nature of the T-B cell cooperation was further characterized with experiments which through the use of chromosomal markers showed that syngeneic or semiallogeneic T and B-cells would more effectively restore immunological responsiveness than allogeneic or xenogeneic T and B-cell combinations (Miller and Mitchell, 1968; Mitchell and Miller, 1968). It was further reported that lymphocyte populations were sensitive to 1000R *in situ* x-irradiation and importantly viable thoracic duct or thymus cells were required for successful immunological reconstitution (Miller and Mitchell, 1968; Claman et al., 1968).

The need for a T-cell is not exclusive to the humoral response to heterologous erythrocytes since the majority of widely used antigens, for example serum proteins, are T-cell dependent (Taylor, 1969; Chiller et al., 1970; Miller and Sprent, 1971). T-cells are also required to initiate humoral antibody responsiveness to defined hapten-carrier conjugates which has been adequately documented by several investigators (Katz et al., 1970; Paul et al., 1970; Mitchison, 1969; Benacerraf et al., 1967). These hapten-carrier studies also further characterized the T-cell as a "helper" cell capable of carrier recognition (carrier specific) and the B-cell apparently exhibits or possesses hapten specificity. The development of *in vitro* lymphocyte culture methods by Mishell and Dutton (1967) and Marbrook (1967) made possible the *in vitro* demonstration of T and B-cell cooperation (Mosier and Coppleson, 1968; Munro and Hunter, 1970; Hirst and Dutton, 1970; Schimpl and Wecker, 1971; Kettman and Dutton, 1971; Katz et al., 1971).

Differential Characteristics of T and B Cells

In addition to the unique functional and maturational differences, there are a number of other markers and activities which can be used to select for or distinguish between T and B cells. A partial list of these characteristics is presented in Table 1.

Table 1.--Pertinent distinguishing features of B- and T-lymphocytes

Parameters	B-lymphocytes	T-lymphocytes
1. Surface Ag markers	B-cell Ag	θ, TL & Ly Ag
2. Surface Ig determinants	High density of Ig (10^5 molecules)	Not really detectable (10^3 or absent)
3. Peripheral localization (as % of lymphocytes)		
a) Blood	10% or less	90% or more
b) Thoracic duct	15-20%	80-85%
c) Lymph node	25%	75%
d) Spleen	60-65%	35-40%
4. Functional sensitivity to x-irradiation	sensitive	relatively sensitive with respect to helper function, cytotoxic activity & delayed type hypersensitivity

Table 1 (continued)

Parameters	B-lymphocytes	T-lymphocytes
5. Functional sensitivity to corticosteroids	sensitive (peripheral) resistant (marrow)	sensitive population (95%)-cortical resistant population (5%)-medullary resistant population is capable of most T-cell functions
6. Response to Ag	AFC (usually T-dependent) not specifically involved in cell mediated immunity	ARC-recognition and binding of Ag, mitotic proliferation, regulatory influence on B-cell but no Ab synthesis, cell mediated immunity
7. Mitotic response to:	Lipopolysaccharide and poke weed mitogen	Phytohemagglutin and Concanavalin A
8. Antigen receptor for:	Hapten	Carrier

Surface antigens. The bulk of the work done in the characterization of the variations in lymphocyte surface antigens has been accomplished with the murine system. Mouse T-lymphocytes exhibit theta (θ) and Ly- alloantigens (Schlesinger and Yron, 1970; Raff and Wortis, 1970; Kisielow et al., 1975). The majority of the lymphocytes in the thymus of some inbred strains may also express a thymus lymphoma (TL-) antigen (Schlesinger, 1972). It is generally believed that the differences in surface antigens may represent the various stages of lymphocyte differentiation and transformation to immunocompetence under the influence of the thymus. The first step is most likely the commitment of the stem cell to the obligatory level of the prethymic cell (Miller, 1975). Under thymic influences, the prethymic cell undergoes transitional changes in surface antigens (Boyse and Old, 1969). The majority of the cells present in the thymus have a high concentration of θ and TL-antigens and are not competent in initiating immunological events (Schlesinger, 1972). A minority of the thymocytes are immunocompetent and are characterized by the loss of demonstrable TL- antigen and a much lower density of θ-antigens, a property shared by the mature, peripheral, recirculating T-cells (Blomgren and Andersson, 1970).

T-cells can also be categorized on the basis of their longevity. Raff and Cantor (1971) distinguished thymus-

derived lymphocytes as T_1 cells (short-lived with high θ antigen density) and T_2 cells as long-lived with low θ activity. It was suggested that antigenic stimulation provoked the transformation of immature T_1 cells into mature T_2 cells which are responsible for all memory and effector functions. The concept of immaturity and immunocompetence for the T_1 cell, however, has recently been questioned (Schlesinger, 1972; Shortman and Jackson, 1974; Cohen and Gershon, 1975). Recent studies suggest that the T-cell antigens serve in a functional capacity to direct the "homing" characteristics and the responsiveness to mitogens exhibited by the various T-cell subpopulations (Schlesinger et al., 1975).

B-lymphocytes are readily characterized by the presence of demonstrable membrane associated immunoglobulins (Raff and Wortis, 1970). These cell surface immunoglobulins have been shown to serve as receptors for the recognition of antigens (Sulitzeanu, 1971). Similar cell surface immunoglobulins may also function as the carrier-recognition receptor on the T-cell, but are present in too few numbers to be readily detectable (Roelants and Ryden, 1974). Distinct B-cell antigens, other than immunoglobulins, also have been identified on thymus-independent lymphocytes (Raff et al., 1971; Laskov et al., 1973). There is also evidence for subsets of B-lymphocytes, since plasma cells possess distinct

antigenic determinants (Laskov et al., 1973) and there is evidence for short-lived (3-5 days) and long-lived (weeks-months) B-cells (Röpke et al., 1975).

Antilymphocyte serum. Antilymphocyte serum(ALS) has been defined as the product obtained when lymphoid cells or lymphoid cell fractions from animals of one species are injected into an animal of another species. ALS has been used in vivo as an immunosuppressant for both CMI and humoral antibody responses and it appears to have a selective action on T-cells. The action of heterologous ALS has been equated with that of the more specific antisera to thymocytes (ATS) in that both seem to exert their effect primarily on the long-lived, recirculating T_2 populations (Lance et al., 1973; Araneo, 1975). Heterologous anti-θ antisera, on the other hand, seems to select for the short-lived T_1 population (Stobo and Paul, 1972; Olsson and Claësson, 1973). ALS or antisera with narrower specificity (i.e., anti-θ) can be used in vitro to characterize lymphoid tissues for the presence and position of T-cell populations by employment of either the complement-mediated cytotoxicity assay or the florescent label tagged antisera. Anti-immunoglobulin and anti-B-cell antigen anti-sera have been used similarly for the detection or elimination of B-cells (Kincade et al., 1970; Raff and Wortis, 1970).

Susceptibility to x-irradiation. It has been known for some time that the immune system is particularly susceptible to irradiation, a fact which, as discussed earlier has provided experimental models for adoptive transfer studies. The capacity of host lymphocytes to engage in immunological activities is abrogated by irradiation, thus allowing for the immunological expression of infused donor cell populations. At first it was thought that both T and B-cells were equally radiosensitive, but it now has become apparent that the T-cell is functionally more resistant to x-irradiation than is the B-cell.

Katz et al. (1970) demonstrated that carrier-primed lymphocytes (T-cells) after having received high doses of *in vitro* x-irradiation were still capable of performing a helper function in enhancing secondary anti-hapten responses in syngeneic guinea pigs. The B-cells responsible for anti-hapten antibody production were, however, functionally destroyed by the same x-irradiation dose.

It was also reported that "educated" helper T-cells were radioresistant to high levels of x-irradiation and could initiate *in vitro* primary responses to SRBC or trinitrophenyl (TNP)-erythrocytes (Hirst and Dutton, 1970; Kettmann and Dutton, 1971). Exposure of carrier (SRBC)-primed spleen cells (mixture of T and B-cells) to high doses of *in vitro* x-irradiation abrogated their ability to develop an antibody

response (B-cell), but did not affect their ability to act in helper capacity (T-cell) in the anti-hapten (TNP) response with normal spleen cells. It was further demonstrated that this radioresistant helper cell was, in fact, a T-cell (Vann and Dutton, 1972). Observations of other investigators have been consistent with the interpretation of a radioresistant T-cell involved both in humoral (Munro and Hunter, 1970; Osaba, 1970; Goldie and Osaba, 1970; Haskill et al., 1970) and cell-mediated immunity (Möller and Möller, 1965; Asherson and Loewi, 1967; Feldman, 1968). It, therefore, seems likely that the expression of T-cell function does not require proliferation.

Corticosteroids. Like irradiation, cortisone has a general suppressive effect on immune responsiveness when administered in large doses to experimental animals. There is an accompanying involution of the lymphoid structures, with the most obvious atrophy occurring in the thymus, where approximately 95% of the total thymic population is depleted by cortisone (Ishidate and Metcalf, 1963; Andersson and Blomgren, 1970). The cortisone resistant thymic population was shown to reside primarily in the thymic medulla, while the corticosteroid sensitive cells are found in the cortex. The cortisone resistant cells have been equated with the T_2 population and the sensitive cells with T_1 (Raff and Cantor, 1971).

Levine and Claman (1970) reported that spleen cells from cortisone treated donors exhibited significantly less ability to adoptively transfer anti-SRBC responsiveness to x-irradiated recipients; whereas, BM cells from cortisone treated animals were perfectly capable of cooperating with thymocytes from non-treated animals. Further studies revealed that adult thymectomized, irradiated, BM reconstituted mice could be restored to humoral antibody responsiveness with infusion of cortisone resistant, syngeneic thymus cells (Cohen and Claman, 1971; Andersson and Blomgren, 1971). It was also shown that thymocytes from cortisone treated donors could restore the <u>in vitro</u> primary anti-SRBC response to a population of mouse spleen cells treated with anti-θ antiserum (T-cell depleted) (Schimpl and Wecker, 1971).

These experiments suggest that T-helper cells are functionally resistant to cortisone. B-cells which have peripheralized are cortisone sensitive; whereas, those which remain in the bone marrow compartment (stem cells) appear to be cortisone resistant. This resistence may be due to the fact that the drug-dose administered was too low to reach the sequestered cells in the bone marrow, rather than reflecting a variation in sensitivities. Cortical T-cells are sensitive to cortisone treatment and are presumably functionally immature with respect to helper function. Medullary T-cells are cortisone resistant and are apparently functionally immun-

ocompetent and equivalent to the fully differentiated, peripheralized T_2 population.

Helper Function (ARC) in Rabbits

Most of the work which established or assigned helper function to the T-cell has been done in either mice or rats. It has been suggested that in the rabbit the cell responsible for helper function may not reside in the thymus-derived population, but rather, in the bone marrow (Abdou and Richter, 1969; Singhal and Richter, 1968; Richter and Abdou, 1969). These conclusions have been based on several different experimental approaches.

Singhal and Richter (1968) reported that, unlike that of other animal species, BM cells from normal adult rabbits were shown to respond with blastogenesis and tritiated thymidine uptake when incubated with various protein antigens in vitro; whereas, lymph node, spleen and thymus cells did not respond in the same manner. Mond and Thorbecke (1972) contradicted this study by demonstrating that normal mouse bone marrow gave a similar proliferate response to antigen as did normal rabbit bone marrow.

Abdou and Richter (1969) exposed nonthymectomized adult rabbits to 800R (surface incidence) total body irradiation with a cobalt-60 source. This treatment destroyed the capacity of these animals to produce splenic anti-SRBC PFC's upon immunization, however, responsiveness

could be restored to normal PFC levels by reconstituting these animals with normal allogeneic BM cells alone. They further reported that BM cells from donors which had been immunized 24 hours previously with SRBC were unable to restore anti-SRBC responsiveness in x-irradiated recipients. From this the investigators concluded that the ARC is BM-derived and that they peripheralize subsequent to antigenic stimulation. Ozer and Waksman (1972) and Landry (1974) found that if a higher x-irradiation dose (900R mid-line, roughly equivalent to 1200R surface) were used, then reconstitution with a single cell population (BM, appendix or thymus) was not sufficient for restoration of anti-SRBC responsiveness, even when recipients received bone marrow cell infusion of 50×10^8. Immune responsiveness could, however, be restored when thymectomized, x-irradiated recipients received a combination of appendix cells and thymocytes which totaled 50×10^8 cells (Ozer and Waksman, 1972). It was suggested that the lower irradiation dose used by Abdou and Richter was selectively depleting the B-cells while leaving the more radioresistant T-cell responsiveness intact.

Richter and Abdou (1969) further suggested that the AFC resides somewhere other than the BM and that the pre-AFC population is radioresistant. Bone marrow cells (5×10^8) harvested from rabbits which exhibit one lymphoid cell

allotype were infused into irradiated (800R surface) recipients which exhibit a different allotype, and the recipients were immunized with SRBC. The splenic PFC expression could be eliminated *in vitro* if the spleens were treated with antisera directed toward the recipient allotype marker; whereas no dimunition in splenic PFC expression was seen when anti-donor allotype serum was used. These data were interpreted and it was suggested that the ARC is present in the infused population of the donor BM cells and that the x-irradiated host provided the relatively radiation resistant AFC.

The interpretation of the above may lose some validity in light of a recent report by Pilarski and Cunningham (1975). They found that lethally irradiated mice could mount a substantial immune response to heterologous RBC under certain circumstances. The response of the host, which may constitute 100% of the total response, occurred only if nucleated cells were transferred. It was optimal with low doses of BM cells (as opposed to spleen cells) and when suboptimal irradiation doses were used. These results suggest that a population of radiation-resistant B-cells exist which can be stimulated to both proliferate and synthesize antibody in the presence of small numbers of nucleated cells. Richter utilized both a low dose of irradiation (800R surface or 400R midline) and a lower BM

reconstituting population (5×10^8 BM cells) than Ozer and Waksman (900R midline and 50×10^8). Therefore, the host immune response in Richter's experiments may actually represent the proliferation of a resistant B-cell population under the influence of viable nucleated BM infusions.

Evidence for a Suppressor T-cell Population

Various different experimental approaches have revealed the probable existence of a lymphoid suppressor cell. Extensive studies concerned with antigen-induced-suppression (antigenic competition) have revealed the phenomenon to be suppressive rather than competitive in nature. Selective elimination of T-cells has shown that they may exert a suppressive effect which is most dramatic on studies done with T-independent antigens.

Antigenic competition. Antigenic competition is a phenomenon which can be expressed when one antigen upon injection causes the suppression of the immune response to a second unrelated antigen. This phenomenon has recently become more appropriately termed antigen-induced-suppression (AIS) (Kerbel and Eidinger, 1971). There have been and still are a number of different theories as to the immunological events surrounding AIS which have been thoroughly reviewed (Taussig,1972; Pross and Eidinger, 1974). It is the intention of this author to deal primarily with the most

widely accepted explanation as to how AIS occurs, namely T-cell induced suppression.

A number of parameters and conditions under which AIS may occur have been documented. It is generally accepted that AIS can be optimally evoked when (1) the two antigens (priming and test) are injected into the same site (Adler, 1957; Eidinger et al., 1971); (2) the priming or suppressing antigen is given in a relatively higher concentration than the test or suppressed antigen (Brody and Siskind, 1969; Dukor and Dietrich, 1970; Eidinger et al., 1971; Thorne, 1972); (3) the priming antigen precedes the test antigen by one to seven days (Brody and Siskind,1972; Eidinger et al., 1968; Hanna and Peters, 1970; Möller and Sjöberg, 1970; Radovich and Talmage, 1967; Thorne, 1972), and (4) the priming antigen must be a T-dependent antigen (Eidinger et al., 1971; Möller, 1971; Sjöberg and Britton, 1972).

Radovich and Talmage (1967) first made the suggestion that a soluble suppressor factor (secreted by sensitive T-cells) may be the vehicle responsible for antigenic competition. Horse erythrocytes (HRBC) were chosen as the priming antigen and sheep erythrocytes (SRBC) as the test antigen, since these two erythrocytes have been shown to lack common antigenic determinants and HRBC had been shown to markedly suppress the anti-SRBC response in mice.

Lethally irradiated mice were reconstituted with varying doses of spleen cells from nonimmunized donors or from donors immunized with HRBC two days prior to transfer, and the recipients simultaneously immunized with SRBC. The spleens were removed from the recipients six days later and assayed for SRBC PFC numbers. It was found that as the spleen cell dose increased from the HRBC-primed donors, the splenic PFC numbers in the recipients decreased. On the other hand, an increase in the number of infused spleen cells from nonimmunized donors resulted in increased numbers of PFC in proportion to the number of spleen cells infused.

Similar results were obtained by Möller and Sjöberg (1970) and Waterston (1970) with HRBC-primed donor cells and a SRBC test antigen. By adoptive transfer of HRBC-primed spleen cells to irradiated mice followed several days later by SRBC immunization, it was shown that the number of ARC for SRBC responsiveness was not affected. However, when normal "non-primed" spleen cells were adoptively transferred to an irradiated recipient which had been previously primed with HRBC, there was marked suppression to SRBC given at the same time.

Brody and Siskind (1969) have studied haptenic competition in rabbits. They observed that antigenic competition would occur even if the competing haptens were conjugated

to homologous or heterologous carrier molecules. This argued against the competition for a limited number of antigen sensitive cells (with mono specificity) and did suggest that hapten specific AFC were being suppressed. They also reported that there was no change in antibody affinity which is in contrast to the nature of suppressed antibody responses observed during immunological tolerance where there is an obvious decrease in antibody affinity. They found that competition occurred only if the antigens were injected so that they came under the immunological influence of the same regional lymph nodes.

Fauci and Johnson (1971a), in contrast, observed that the regional lymph node response to p-arsanilic acid-keyhole limpet hemocyanin (Ars-KLH) injected into the foot pads on one side of the experimental rabbit was markedly suppressed by the contralateral foot pads. They interpreted these data as evidence for a circulating inhibitory factor. They also showed that ip preimmunization with KLH (carrier alone) caused a marked suppression of the PFC responses to both Ars-KLH and TNP-KLH when injected into the foot pad three weeks later (Fauci and Johnson, 1971b). This finding is similar to the suppression observed in the "preemption" experiments reported by O'Toole and Davies (1971) during which the ip injection of SRBC caused a suppression in anti-SRBC responsiveness in the RLN draining the site of a

subsequent subcutaneous (sq) injection of SRBC.

Gershon and Kondo (1971a) clearly demonstrated that antigenic competition between SRBC and HRBC was thymus dependent. Mice deprived of T-cells (adult thymectomized, irradiated, BM-reconsituted) were unaffected in their response to HRBC after being primed with SRBC two to four days earlier. The reconstitution of these animals with graded doses of thymocytes revealed that the greater the number of T-cells infused, the greater antigenic competition to the test antigen. In a second study, Gershon and Kondo (1971b) demonstrated that antibody production to the priming antigen is not an important factor during antigenic competition. They showed that high titer anti-SRBC antibody infused into test animals in concentrations sufficient to suppress the antibody response to SRBC (primer Ag) would not alleviate the AIS of the test (HRBC) antigen.

Enhancement by depletion of T-cells. ALS (ATS) has been used to suppress the humoral response to a number of antigens (James, 1969; Lance et al., 1973). Presumably, suppression occurs through the elimination of the helper T-cell necessary for antibody responsiveness (Martin and Miller, 1968). It has become apparent that there exists a group of antigens which are capable of provoking normal antibody responses even in the absence of T-cells. These antigens characteristically have molecular structure which

consists of repeating identical haptenic units arranged in a linear sequence (Katz et al., 1969) and, in addition, humoral immune response to these antigens is characterized by, primarily, the IgM class of immunoglobulins (Britton and Möller, 1968; Katz et al., 1969).

Baum et al. (1969) reported that rats pretreated with the IgG fraction of ALS showed a marked suppression of response to SRBC, however, the response to KLH was 16 times greater than that of non-treated animals. During a study on the effects that T-cell depletion had on subsequent immunizations with various antigens, Kerbel and Eidinger (1971) reported similar results. They found, with ALS treatment or with ALS treatment in combination with adult thymectomy, that the antibody response to SRBC was suppressed; whereas, the response to either polyvinylpyrrolidine (PVP) or KLH was significantly enhanced. The enhanced PVP response continued through 25 days. The KLH response was enhanced through 15 days, but then began to show some suppression during the last 10 days. The enhanced anti-KLH response was restricted to IgM and the subsequent suppression occurred during the shift from IgM to IgG synthesis. The response to PVP was entirely IgM. These data were interpreted to suggest that the early anti-KLH response was T-independent and like the response to PVP is normally suppressed by the T-cell population. The shift to IgG

synthesis during the KLH response is dependent upon T-cell helper function which had been removed earlier.

Armstrong et al. (1969) similarly implicated the T-cell subpopulations playing a suppressor role since the response to thymus-independent antigens such as PVP or polymerized flagellin (POL) in T-cell deprived mice could be suppressed with the infusion of thymocytes. Baker et al. (1970a) showed that the primary PFC response to pneumococcal polyssacharide SSSIII (T-independent) could be enhanced 10-fold when the mice were treated with ALS. Enhancement could be suppressed by the infusion of thymocytes (Baker et al., 1970b).

Okumura and Tada (1971a and 1971b) showed T-cell suppression of the hemocytotropic antibody (HTA) response in rats immunized with DNP-Ascaris (As), (a T-dependent response). Adult rats which were thymectomized or splenectomized three to 10 days before primary immunization with DNP-As showed greatly enhanced and prolonged HTA titers (Okumura and Tada, 1971a). If the rats were neonatally thymectomized, they showed marked reduction in their capacity to develop anti-DNP HTA responses to DNP-As. Adoptive transfer of thymocytes from donors which had been hyperimmunized with As terminated the preexisting anti-DNP HTA production in recipients.

Biological nature of the T-suppressor cell. Two

questions are currently being pursued by investigators interested in suppressor function. Is suppressor activity the function of a distinct subpopulation of T-cells or is it an antigen driven switch of helper function to suppressor function in the same cell? Is suppression mediated by some soluble factor or factors elaborated by T-cells or is there a requirement for cell-to-cell contact?

The bulk of current research in this area has indirectly supported the existence of a suppressor subpopulation in the T-cell compartment. When density purification with velocity sedimentation was used with spleen cells, a cell fraction was obtained which was strongly inhibitory toward the responsiveness of the other, normally responsive, spleen cell fractions to SRBC (Haskill et al., 1971; Haskill and Axelrad, 1972). The suppressive function of this cell fraction was sensitive to treatment with anti-θ and complement.

Dutton (1972) presented evidence which suggested that the mitogen concanavalin A (Con A) stimulates at least two different cell populations. The first cell was responsible for an inhibitory effect on anti-SRBC responsiveness. This cell population was shown to be radiosensitive, short-lived and sensitive to treatment with anti-θ antiserum characteristic of the T_1 population as described by Raff

and Cantor (1971). The other mitogen responsive cell population could not be identified on the basis of the data presented, but could be substituted for, and exhibited the helper T-cell function in B-cell response.

Scavulli and Dutton (1975) selected for both the Con A-induced suppressor cells and the Con A-induced stimulator cells. They found that spleen cells with inhibitor activity could be generated by incubating spleen cells in the presence of Con A for 48 hours followed by exposure to 1000R irradiation. If the procedure were reversed and the cells were irradiated first and then incubated with Con A, a cell population with stimulator activity was selected for. Both of these populations were free of B-cell activity which had been eliminated by the irradiation step. Spleen cells were harvested from congenitally athymic (nu/nu) mice cultured and challenged with SRBC and the immunological responsiveness was measured after the addition of varying numbers of stimulatory and/or suppressor cell populations. It was found that suppressive effects could be reversed by the addition of stimulatory cells or by pretreatment of the inhibitor population with anti-T-cell serum and complement. These results are compatible and re-inforce the concept that two distinct populations of T-cells exist which mediate responsiveness either by stimulation or suppression.

Eidinger and Pross (1972) reported that suppressor cells were cortisone-resistant thymus cells which were sensitive to irradiation. In contrast Weksler, Shell and Siskind (1974) reported that suppressor cells were cortisone sensitive and anti-thymocyte serum resistant. Cohen and Gershon (1975) have suggested that a cortisone-sensitive T-cell population may play a regulatory role on the highly reactive cortisone-resistant thymocytes.

Wu and Lance (1974) have suggested that there exists a subpopulation of cells normally resident in the thymus and spleen, but not in the lymph nodes, which can suppress the humoral response to SRBC. These T-cells are spleen-seeking and require the microenvironment of the spleen for the expression of their suppressive function. Their results further suggest that the suppressor cell is cortisone-resistant and relatively short-lived (two to four weeks).

Gershon et al. (1974) also suggested an immunoregulatory role for spleen-seeking thymocytes. These investigators examined the reaction (DNA synthesis) of infused parental thymocytes to the antigens in the spleen and lymph nodes of irradiated F_1 mice recipients. They removed the spleen-localizing fraction of thymocytes by splenectomizing recipients three hours after cell injection. Wih high thymocyte doses splenectomy resulted in elevated lymph node

response, which can be reversed (suppressed) by reinjection of spleen cells.

Kamin et al. (1974) reported that the appendix as well as the spleen and Peyer's patches housed cells with suppressor activity, which could be eliminated by complement cytolysis in the presence of ATS. Suppressor activity could be enhanced by adsorption of the B-cells in the appendix to nylon wool columns thus enriching the T-cell population. They suggested that suppression may be one of the primary functions of the appendix in rabbits.

Suppressor T-cell function declined in NZB strain mice with age and these mice expressed an auto-immune disease more frequently with age (Barthold et al., 1974). It was found that the T-independent PFC response to SSSIII increased with age in NZB mice; whereas, there was no apparent age associated increase in responsiveness observed in non-autoimmune BALB/c mice. The SSSIII response in 10 month-old NZB mice could be suppressed by infusion of thymocytes from four week-old NZB donors. Evidently, suppressor activity is a function of a separate T-cell subpopulation since the helper and effector functions in these mice were still apparent at two to three months, whereas suppressor function was absent. There was still a very high response to SRBC in four month-old NZB mice.

Thomas et al. (1975) characterized a soluble sup-

pressor factor released by immune spleen cells *in vitro*. The addition of low numbers of ovalbumin (OVA) immune spleen cells to SRBC-immune spleen cells (ISC) in a Mishell and Dutton (1967) tissue culture system reduced the number of anti-SRBC PFC in the presence of low concentrations of soluble OVA. The suppressor population was found to be irradiation sensitive (750R) and sensitive to the treatment of anti-θ and complement. Inhibition would occur even when the two ISC populations were separated by a cell-impermeable membrane and the soluble inhibitor could be recovered from the chamber opposite the OVA-ISC system. The suppressor factor was shown to be sensitive to trypsin treatment and resistant to up to 70° C for 30 minutes. In a sucrose gradient the M.W. was shown to be 55,000-60,000 daltons. This factor was distinct from the helper factor (Rubin and Coons, 1972; Gisler et al., 1973; Schimpl and Wecker, 1972; Yu and Gordon, 1973) which had been shown to be sensitive to treatment at 70° C for 30 minutes. Thomas et al. (1975) suggested that these factors are elaborated by separate T-cells since helper factor production was in part irradiation resistant, whereas the suppressor factor function was irradiation sensitive. These findings further implied that cellular proliferation is required for suppressor factor, but not for helper factor production.

Chapter 2

MATERIALS AND METHODS

Experimental Animals

Rabbits. Animals used in these studies were randomly bred, New Zealand White rabbits weighing 1.5-1.8 kilograms (8-10 weeks old) obtained from a closed colony maintained in this laboratory. The rabbits after weaning were housed in stainless steel cages, fed Purina or Red Circle rabbit pellets and given water ad libitum.

Sheep. Pastured adult female western sheep from the LSU sheep farm (Ben Hur Road, Baton Rouge, La.) were used as red cell donors. Sheep blood was collected at approximately 4 week intervals from the jugular vein, into an equal volume of Alsever's solution (Campbell et al., 1963) and stored at 4° C until needed. The same red cell lot was used for both immunization and for subsequent immunological assays.

Horses. Welsh ponies penned at the Diseased Animal Center on the LSU campus were used for both immunization in the production of anti-rabbit thymus serum and as red blood cell donors. Horse blood was collected at approximately 4 week intervals from the jugular vein into an

equal volume of Alsever's solution and stored at 4° C until needed.

Erythrocyte Antigen

Preparation of the erythrocyte antigens. When needed, approximately 7 ml of RBC-Alsever's mixture was diluted to 15 ml with sterile 0.15 M NaCl (saline) or appropriate diluent and sedimented, in a 15 ml conical centrifuge tube, at 1200 rpm for 10 minutes at 4° C in a model PRJ-refrigerated International Centrifuge (International Equipment Co., Needham, Massachusetts). The supernatant fluid and excess cells were aspirated by means of suction to the desired packed cell volume. The RBC sediment was washed three times with the appropriate diluent and finally resuspended to the desired concentration.

For immunization, a 10 percent suspension of SRBC and a 20 percent suspension of HRBC were prepared in sterile saline. One ml of the RBC suspension was injected iv via the rabbits' marginal ear veins.

Complement

Source and preparation of complement. Complement used in these studies was lyophylized guinea pig serum (Hyland Co., Division of Tratenol Laboratories Inc., Costa Mesa, California) obtained from the Baton Rouge General Hospital serology laboratory. The reconstituted stock was

stored at -20° C in 1 ml aliquots in serum vials. Prior to use, the complement was thawed in an ice bath and diluted at 4° C in modified barbital buffer (Table 2) when used in serological assays or in Hank's balanced salt solution (HBSS, Table 3) when used for either plaque-forming cell determinations or cytotoxic assays.

Collection and Preservation of Rabbit Serum

A minimum of 3 ml of blood was collected from the central artery of the ear using a 22 gauge needle and a 10 ml plastic syringe. The blood was allowed to clot at room temperature for 30 minutes, then freed from the tube by ringing the clot with a wooden applicator stick and stored at 4° C. The serum was then decanted to 4 ml serum vials and stored at -20° C until needed for serological assay.

Serological Assay for SRBC Hemolysin Activity

Serum samples were thawed and the native complement activity was inactivated in a 56° C water bath for 30 minutes. Microtiter techniques and equipment (Cooke Engineering Co., Alexandria, Virginia) were used to determine hemolysin titers. U-shaped wells in the microtiter plates were filled with 0.025 ml of modified barbital buffer (Table 2) using a 0.025 ml micropipette. The 0.025 ml diluters were cleaned in distilled water, heated to incandescence, sub-

Table 2.--Modified barbital buffer used in hemolysin microtiter assay of serum

Constituents	Concentration grams/liter
5,5 Diethylbarbituric Acid (Barbital; Veronal)	2.8750
Sodium 5,5 Diethylbarbiturate (Barbital sodium; Veronal sodium)	1.8750
Calcium chloride, dihydrate	0.1103
Magnesium chloride, hexahydrate	0.5083
Sodium chloride	42.5000

Procedure

Dissolve the barbital in 250 ml of hot distilled water (near the boiling point). Then successively add and dissolve the remaining reagents in the hot water. Store at refrigerator temperatures. This is the stock. For use dilute 1 part of the stock solution with 4 parts of distilled water and chill. The final pH of the buffer should be 7.3 to 7.4. Prepare fresh dilution each week.

Table 3.--Hank's balanced salt solution used in collecting and resuspending the cells for the PFC assays

Constituents
Flask 1. NaCl, 8 g; KCl, 0.4g; $Na_2HPO_4.7H_2O$, 0.189 g; KH_2PO_4, 0.05 g; glucose, 2 g; gelatin, 1 g in 700 ml of boiled redistilled water.
Flask 2. $CaCl_2.2H_2O$, 0.265 g in 100 ml boiled redistilled water.
Flask 3. $MgSO_4$, 0.09772 g in 100 ml boiled distilled water.
Flask 4. $NaHCO_3$, 0.34 g in 100 ml boiled redistilled water.

Procedure
1. Autoclave flasks 1, 2, 3, and 4 for 10 minutes.
2. When ready to use, mix the contents of flasks 2, 3, and 4 into 1.
3. Check pH (range from 7.2 to 7.6).

merged in distilled water, and calibrated by blotting dry on a calibration blotter. The serum samples were drawn into the diluters by capillary action and the serum loaded diluters were placed in the first well of each respective row. The serum and buffer were mixed using a microtiter diluting handle and 0.025 ml of each dilution was carried to the second well of each row. This procedure was repeated through 12 wells or more, as the titer dictated, thus affecting a two-fold serial dilution. Next 0.025 ml of a 0.5 percent SRBC suspension was added to each well and mixed with the diluted sera by gentle tapping of the plates. Stock complement was diluted 1:15 and added in 0.025 ml volumes to each well. After thorough mixing, the plates were recorded as the reciprocals of the greatest $\log_2$ dilution of serum which showed complete hemolysis.

Assay for Splenic Plaque Forming Cells (PFC)

Collection of spleen cells. Rabbits were exsanguinated by cardiac puncture and death was insured by the injection of 30 ml of air directly into the heart. The peritoneal cavity was opened and the spleen was removed and trimmed free of excess connective tissue and fat. The organ was immediately transferred to a stainless steel cup with a sieve insert with a maximum pore size of 74 micron.

The spleen was minced (to a single cell suspension) with scalpel blades to free individual spleen cells and

these cells were washed with approximately 40 ml cold HBSS through the sieve into a 250 ml beaker immersed in an ice bath. The lymphoid cell suspension was centrifuged in a thick-walled 40 ml centrifuge tube at 1200 rpm for 10 minutes at 4^0C. The supernatant fluid was discarded and the cells were resuspended in 15 ml HBSS, transferred to a 15 ml calibrated conical centrifuge tube and sedimented.

A 10% spleen cell suspension (volume/volume) which contained approximately 10^8 cells was prepared in HBSS (stock suspension) from which appropriate dilutions were made (Landry, 1974).

Modified method for enumeration of splenic PFC numbers. A modification of the direct PFC assay of Jerne and Nordin (1963) was used (Landry, 1974). Briefly a mixture of 0.05 ml of each spleen cell dilution (i.e., 10^8, 10^7, 10^6 cells) and 0.05 ml of a 7.5% suspension of erythrocytes in 0.4 ml of tempered (45^0C) 0.5% agarose was layered onto each quadrant of a Pyrex Petri dish cover precoated with 0.1% agarose. Next, the surface of the Petri dish was gently flooded with a 1/32 dilution of the stock guinea pig complement. The dishes were placed on trays, covered with aluminum foil to maintain humidity and prevent drying and incubated at 37^0C for two hours. The plaques were counted under indirect lighting and the totals were corrected to

give the number of PFC per million spleen cells and per whole spleen (Landry, 1974).

Reconstitution Studies

X-irradiation Procedure. All x-irradiation studies were performed with a Phillips-Norelco x-ray machine housed in the Nuclear Science Department on the LSU campus. Rabbits were retained under the source with cloth restraints and their behavior was monitored during irradiation with closed circuit television. Animals received an internal dosage of 60 R per minute at a machine setting of 300 kV and 10 mA through a 2 mm aluminum plate at a target distance of 55 cm. This dose rate was previously determined using a "phantom carcass" and a Victoreen monitor (Victoreen Instrument Co., Cleveland, Ohio) as described by Landry (1974).

Pre and post x-irradiation care of animals. Rabbits were injected intramuscularly (im) with 0.5 ml of antihistamine (Inactihist L.A., 7.5 mg chlorpheiramine maleate, Diamond Laboratories, Inc., Des Moines, Iowa) at least 30 minutes prior to irradiation and then were injected im with 0.4 ml Combiotic (200,000 units penicillin and 250 mg streptomycin; Charles Pfizer and Co., Inc., New York, New York) immediately after x-irradiation. The antihistamine was necessary to prevent death apparently due to anaphylaxsis from non-specific release of histamine, which occurred in approximately 1 of 4 rabbits about 90 minutes following

x-ray treatment.

Individual animals were placed in steam cleaned cages following x-irradiation.

Thymectomy. Rabbits which weighed between 1000 and 1500 grams (approximately 7 to 8 weeks old) were more conducive to successful thymectomy due to less vascularization of the chest, little or no fat in and around the thymus and enhanced post surgery recovery. The chest area was shaved from the neck to the epiphisternum using a staight-edged razor and soap. The area was then cleaned using Phisohex and water applied with a sterile gauze. One ml of 2.5 % sodium thiopental was administered iv and up to 2.0 ml was given throughout the surgical period as needed. An incision was made through the dermis and muscle layers to the immediate left of the sternum (as viewed from the anterior position) extending from the hyoid cartilage to the level of the fourth rib. The chest cavity was entered by cutting through the first and second ribs at their sternocostal junctions. The muscle layers were cut to the third rib and the opening was gently retracted using stainless steel spreaders. The thymus was removed *in toto* with dog-toothed forceps from its position above the heart. By gentle pulling, the thymus could be separated from its attachment site on the aorta. The musculature and ribs were closed with a single suture line of 0000 gut suture and the dermis was

closed using either 7.5 mm surgical clips or 0000 silk or synthetic suture. The area was cleaned with Phisohex and the rabbits were given 0.4 ml Combiotic im. The rabbits were housed individually in steam cleaned cages and allowed to convalesce at least 7 days prior to further experimental manipulation.

Cortisone treatment of donors. Littermates of the thymectomized, x-irradiated rabbits, which also weighed between 1500 and 1800 grams, were injected intraperitoneally (ip) with 250 mg of hydrocortisone-21-acetate (Sigma Chemical Co., St. Louis, Missouri) suspended in 20 ml of sterile saline. Seventy-two hours later, these rabbits were sacrificed by exsanguination and were used for bone marrow and thymus cell donors. Cortisone treatment destroys immature cortical thymocytes leaving the mature medullary T-cells intact (Andersson and Blomgren, 1970). This treatment effectively reduced the thymus cell population to less than 10% that of an untreated rabbit. Cortisone treatment also destroys the peripheral bone marrow-derived cells (B-cells), but does not appear to significantly affect B-cells present in the bone marrow (Cohen and Claman, 1971; and Levine and Claman, 1970). In this manner a more effective mature T-cell population could be obtained without any obvious significant effect on the B-cell.

Collection of Bone Marrow and Thymus Cells for Reconstitution

Collection and preparation of BM cells. In all experiments littermates were selected for BM-thymus donor-recipient pairings and they were also matched with respect to sex. Never were the reconstituting cell populations of several donors pooled. BM was collected from the femurs and the tibio-fibulae into tissue culture medium 199 containing 500 units of sodium heparin and 10 mg of pancreatic deoxyribonuclease I (Sigma Chemical Co., St. Louis, Missouri) per liter. This procedure was thoroughly discussed by Landry (1974) and with the exception of the addition of DNAase the procedure was unchanged. The BM cells were counted in an hemocytometer and $20x10^8$ nucleated cells in approximately 6ml of TCM 199 were infused through a 22 g needle into the marginal ear vein of the recipient at a rate of 3 ml per minute.

Thymus cell preparation for infusion. In all experiments, if a recipient received both BM and thymus cell infusions, both cell populations were collected from the same donor. The entire rib cage was removed in order to expose the thymus, taking care not to nick any vessels. The trachea was then exposed above the heart, lifted with a dog-toothed forceps and severed. The trachea was then lifted out of the chest cavity and by cutting the dorsal attachment sites the heart, lungs and thymus were removed. The heart was then

opened to drain the blood without contaminating the thymus. The thymus was then dissected free of the trachea and blood vessels and placed in a sterile Petri dish which contained 20 ml of chilled TCM 199 containing heparin and DNAase on an ice bath. When chilled in this manner, the fat congealed allowing the thymus tissue to be distinguihed from the fat which was then teased and dissected from the thymus and discarded. The thymus was then carefully minced with a curved scissors and forceps, freeing the thymus cells. The TCM 199 which contained the thymus cells was removed with a sterile Pasteur pipette and transferred to a 50 ml screw cap conical centrifuge tube. Fresh TCM 199 was added to the thymus tissue and the procedure repeated until 40 ml of thymus cell suspension had been collected in each of two centrifuge tubes. The tubes were shaken vigorously for 2 minutes and centrifuged at 1200 rpm for 10 minutes at 4^{o} C. This procedure further enhanced the separation of thymocytes from fat which could be removed from the top of the supernatant fluid. After the fat and supernatant fluid were decanted, the cells were resuspended in fresh TCM 199 and then passed once through a coarse stainless steel sieve and then through a fine mesh (74 micron) sieve to produce a single cell suspension. The resulting suspension was sedimented again in a sterile 40 ml centrifuge tube at 1200 rpm for 10 minutes at 4^{o} C. The supernatant fluid was

aspirated, the cells resuspended in 15 ml of fresh TCM 199, transferred to a 15 ml conical centrifuge tube and centrifuged at 1200 rpm for 10 minutes. The cells were resuspended in 10 ml of TCM 199 and cell counts were made.

Thymus cell infusion. Rabbits were given 30×10^8 normal thymus cells in the same manner as described for BM infusions.

When cortisone treated rabbits were used as the BM and T-cell donors the entire thymus cell population was mixed in 10 ml of TCM 199 with approximately 20×10^8 BM cells and this mixture was infused as previously described.

Experimental Protocol

It had been previously determined that reconstitution with bone marrow cells alone did not restore immunological responsiveness to SRBC antigens in lethally x-irradiated rabbits (Landry, 1974). The objective of one aspect of this study was to determine if infusion of thymus cells in conjunction with BM cells could restore immunological responsiveness. The experimental design is presented in Figure 2. Since the epithelial cell compartment of the thymus is not significantly affected by x-irradiation, it is conceivable that subpopulations of infused BM lymphoid cells could migrate to the thymus, differentiate and become mature, immunologically competent T-cells under the influ-

ence of the reticuloepithelial structure (Katz and Benacerraf, 1972). Therefore, to avoid this possibility, recipients were thymectomized 1 week prior to x-irradiation and lymphoid cell infusion.

For almost 2 years all attempts to infuse thymus cells met with uniform failure due mainly to the presence of large numbers of lymphoid cells which never leave the thymus. These cells are very fragile and when ruptured (i.e. during centrifugation) they release large amounts of nucleoproteins (Weir, 1973). These nucleoproteins aggregate during infusion and form emboli in the small capillaries which kill the rabbit, often before infusion of the thymus cells can be completed. In order to circumvent this problem, whole thymus implants and ip injections of thymus cells were attempted with little or no success. The addition of DNAase to collection, washing and infusion fluids made it possible to introduce thymus cells iv without the production of emboli. In later experiments cortisone was injected into the donors in an attempt to select for the more mature, competent T-cell population and in this way eliminate the immature T-cells (90%). The infusion of cortisone-resistant T cells plus BM cells significantly reduced death due to embolism.

In all experiments representative animals were sacrificed 7 days following immunization with SRBC and their

spleens were assayed for PFC numbers. Serum hemolysin titers were done for each experimental animal until sacrificed or on selected days 0 through 30 or until they died following exposure to x-irradiation and reconstitution. WBC counts per mm^3 were done on blood samples taken on the 2nd, 3rd, 4th, 5th, and 7th day post BM infusions (Figure 2).

Thymectomized, x-irradiated, BM infused, immunized rabbits. This experiment was designed to determine the capacity of BM cells to reconstitute immunological responsiveness to SRBC in thymectomized, lethally irradiated rabbits. Thymectomized, lethally irradiated (900R) rabbits were infused with 20×10^8 allogeneic (litter mate) bone marrow cells within 4 hours after x-irradiation. They were then immediately immunized with 1.0 ml of 10% SRBC.

Thymectomized, x-irradiated, BM and thymus infused immunized rabbits (T-X-BMT-I). In this experimental group the capacity of BM and thymus cells to reconstitute the immune resposiveness to SRBC was examined. Thymectomized, irradiated rabbits each received the total BM and T-cell population from one cortisone-treated donor litter mate within 4 hours after irradiation. They were immunized with SRBC immediately following infusion.

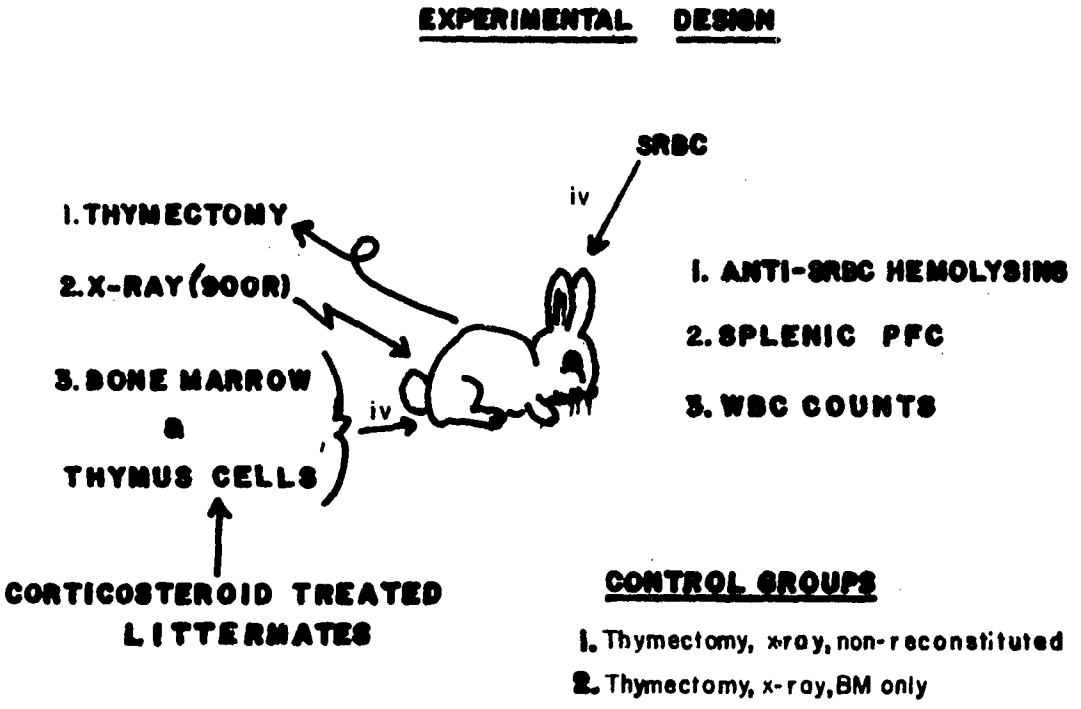

Figure 2. The experimental protocol for the x-irradiation, reconstitution studies.

II. Horse Anti-rabbit Thymocyte Gamma Globulin Studies.

The purpose of the second aspect of this investigation was to determine the effects, if any, on the in vivo administration of anti-T-cell antibody on the immunological resposiveness of rabbits to SRBC.

Preparation of horse anti-rabbit thymus serum(HARTS). Two Welsh ponies received at weekly intervals 3 iv injections of 3×10^{10} New Zealand White rabbit thymus cells suspended in 30 ml of HBSS (De La Nove, Koperstych and Richter, 1972). Two liters of blood were taken from the jugular vein on the 2nd, 3rd and 5th week after the final thymocyte injection. The serum was harvested as previously described and stored in 50 ml aliquots at -20^{0}C.

DEAE Fractionation of HARTS. The IgG fraction of horse anti-thymus serum has been reported to be as effective and less hazardous than whole serum or its ammonium sulfate fraction when used as an immunosuppressant during therapy treatment (Lance, Medawar and Taub, 1973). For this reason the IgG fraction, anti-thymus gamma globulin (ATGG), was used exclusively in these studies. Thirty g of DEAE Whatman DE 23 (Cat. No. 24233) was suspended in 800 ml of 0.5 N NaOH and 0.5 M NaCl, stirred until bubbles were no longer apparent and allowed to settle for approximately 30

minutes. The supernatant fluid was decanted and the DEAE was resuspended in approximately 600 ml of 1 N NaCl. The suspension was stirred, allowed to stand for 30 minutes and the supernatant fluid decanted. The DEAE was washed with 400 ml of 1 M NaCl and then filtered through two thickness of 15 cm Whatman number 1 qualitative filter paper under negative pressure. Approximately 400 ml of 1 N HCl was added to the DEAE and immediately filtered. The DEAE was rinsed once with distilled water, resuspended in approximately 700 ml of distilled water and allowed to stand for approximately 4 hours at 4° C. The slurry was washed and filtered with distilled water until the pH of the filtrate was 7.0. The DEAE was next suspended in 1 liter of 0.01 M phosphate buffer pH 8.0 (Table 4) and allowed to stand over night at 4° C. The slurry was stirred under negative pressure in order to remove trapped air. A glass column (20 x 400 cm) was filled by pouring the slurry down a glass rod into the column. The buffer was allowed to run through the column port as more slurry was added. After the column was filled, an additional 200 ml of phosphate buffer was allowed to run through the column.

A 30 ml aliquot of thawed HARTS was heated in a 56 C water bath for 30 minutes (complement inactivation) and then adsorbed with thrice washed rabbit RBC (0.1ml packed cells/1.0ml serum) in an ice bath for 10 minutes to remove anti-rabbit RBC hemolysin. The serum-RBC suspension was centri-

Table 4.--Phosphate buffer used for the DEAE-chromatography fractionation of horse serum to the IgG component

Stock phosphate buffer:

540 ml 0.5 M Na_2HPO_4
60 ml 0.5 M KH_2PO_4
pH 8.0

For fractionation or dialysis:

20 ml of the stock is brought up to a liter with distilled water.
0.01 M phosphate buffer pH 8.0

For washing the column:

29 grams NaCl is added to each liter of 0.01 M phosphate buffer
0.5 M NaCl in 0.01 M phosphate buffer pH 8.0

fuged at 1200 rpm for 10 minutes and the serum was removed from the RBC sediment. The adsorbed serum was dialyzed at 4 C against 100 volumes of 0.01 M phosphate buffer pH 8.0 which was changed three times at 8 hour intervals. Next, the dialyzed serum was centrifuged at 3000 rpm for 30 minutes at 4 C to remove pseudoglobulin precipitate.

The buffer level in the column was allowed to enter the DEAE bed and then 10 ml of dialyzed horse anti-serum was added, carefully, to the surface of the DEAE so as not to disturb the bed. The serum was allowed to enter the bed, and then the column was filled carefully with buffer. The buffer was allowed to flow from a reservoir through the column at a rate of 1-2 ml per minute. The eluant was collected in 10 ml fractions. Optical density readings were made at 280 nm in a Beckman double beam spectrophotometer. All fractions with an O.D. of 0.5 or greater were pooled. The peak usually started after 50 ml had been collected (5th tube) and was generally complete within the next 30 ml (8th tube). These fractions were pooled and placed in dialysis tubing and concentrated from 30 to less than 10 ml by covering the tubing with polyvinylpyrrolidine or Aquacide II (Calbiochem, La Jolla, California) at 4 C.

Total protein determinations were performed on the concentrated IgG sample by utilizing a modification of the Lowry method (Williams and Chase, 1971). The total protein

concentration of each IgG (ATGG) preparation was adjusted to 10 mg per ml by diluting with phosphate buffer and the molarity of the sample was adjusted to 0.15 with the addition of crystalline NaCl. Normal horse gamma globulin (NHGG) was prepared in the same manner as described for the immune serum described above.

Analysis of ATGG Fractions

ATGG was examined by immunoelectrophoresis against rabbit anti-horse whole serum in order to determine the homogeneity of the IgG fraction.

Preparation of rabbit antiserum to horse whole serum. Horse serum (HS) diluted 1:3 was emulsified in an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan). One ml of the emulsion was injected im into four sites in the upper hind legs of two rabbits. Thirty-six days later HS emulsified in Freund's incomplete adjuvant (Difco Laboratories, Detroit, Michigan) was injected into the rabbit by the same route. Seventy-four days after the first injection the rabbit received an iv injection of 0.5 ml of a 1:3 dilution of HS, 1.0 ml on day 75 and 2.0 ml on day 76. One week later serum samples were collected and used (rabbit anti-HS antiserum) in the immunoelectrophoresis procedure.

Immunoelectrophoresis procedure (Oxoid Apparatus). Table 5 shows the material used in the electrophoresis pro-

Table 5.--Reagents used for the electrophoresis of horse serum and horse anti-rabbit thymocyte gamma globulin in gel and for the immunodiffusion and precipitation with rabbit anti-horse serum.

1. Stock barbital buffer:
   770 ml 0.1 M sodium barbital (20.6 g/l) + 230 ml 0.1 N HCl

   Ionic strength 0.077; pH 8.4

   Stock buffer diluted 1:2 for use in electrophoresis chamber and for agar preparation.

2. Agar:
   1.6% Noble agar in barbital buffer was used as experimental layer on slides.

   0.2% Noble agar in barbital buffer used as adhesive layer on slides.

3. Solution used to stop pattern development:
   2% NaCl in 0.05 M phosphate buffer (0.375 g KH_2PO_4 and 12.6 g $Na_2HPO_4 \cdot 7H_2O$ per liter)

4. Dye solution:
   1 g Ponceau Red + 425 ml 1 M acetic acid + 425 ml 0.1 M sodium acetate.

5. Decolorizing solution:
   2% acetic acid

cedure. A thin layer of adhesive agar was applied to pre-cleaned glass slides using a piece of filter paper as a brush. The agar was dried at 100° C for 5 minutes. A 1.3 mm experimental agar layer was prepared on each slide by applying melted agar (80° C) to the slides with a warmed pipette (2.5 ml per slide). The wells (1 mm in diameter) and trough (2 mm in width) were cut in one operation with a gel punch and the agar plugs were removed from each well. Undilute samples of HS and ATGG were delivered to the outer wells on the same slide. The slides were placed in the electrophoresis chamber and wicks consisting of two layers of 3 MM filter paper were set in place. Electrophoresis was accomplished in 90 minutes with a voltage gradient of 6 volts per cm. The agar was then removed from the longitudinal trough and undilute rabbit-anti-horse-serum antiserum was added with the aid of a three ml syringe and 25 gauge needle. The slides were allowed to develop in a humid environment at room temperature for 24 hours. Next the slides were placed in stopping solution (Table 5) for 72 hours with several changes. Next the slides were washed in several changes of distilled water over a 24 hour period. The wells and trough were then filled with water and a piece of moist filter paper was placed over the agar and the slide was placed in a 37° C incubator until the agar had dried to a thin film. The dried slides were placed in the dye sol-

ution (Table 5) for three hours and decolorized with 2% acetic acid until the background gel was clear. After a final wash in distilled water the slides were air dried.

Photographs were made by using the slide as a negative. It was placed directly in the enlarger head of a Beseler enlarger with 80 mm lens. Ilford no. 4 glossy paper was exposed for 10 seconds at a lens opening of f22, followed by development in the Ilford machine.

Cytotoxic assays of ATGG fractions. Cytotoxic assays were performed according to the method of DeLaNove, Koperstych and Richter (1972). A serial two-fold dilution of ATGG was made in HBSS and 0.1 ml of each dilution was then transferred to duplicate rows of Kahn tubes (10x75 mm). A 0.1 ml suspension, which contained 2-3 x 10^6 normal viable thymocytes per ml, was added to each of the tubes, followed by 0.1 ml of a 1/5 dilution of stock complement. Control tubes were set up in a similar manner except that normal horse gamma globulin (NHGG) was substituted for ATGG. Additional controls consisted of duplicate tubes containing a) 0.1 ml of ATGG diluted 1/2, 0.1 ml of test cells and 0.1 ml of HBSS b) 0.1 ml test cells, 0.1 ml of complement and 0.1 ml of diluent c) 0.1 ml of NHGG (1/2), 0.1 ml test cells and 0.1 ml diluent d) 0.1 ml test cells and 0.2 ml of diluent. All tubes were incubated at 37° C in a water bath for one hour. Next all the tubes were immersed in an ice

bath to stop the action of complement. One tenth ml of a trypan blue solution (4 parts 0.2% trypan blue stock and 1 part 4.25% NaCl stock) was added to each tube. The trypan blue reagent was prepared from stock solutions immediately before needed.

The proportion of stained (dead) or unstained (live) cells was determined by the microscopic examination of 200 cells in a Bright-line hemocytometer under the high, dry objective of a light microscope. Cells were examined two minutes after addition of trypan blue. The cytotoxic index of ATGG was calculated from the following formula:

$$\text{Cytotoxic index} = \frac{DE-DC}{100-DC} \times 100$$

where DE represents the percentage of dead cells in the tube which contained ATGG dilutions and complement, and DC represents the percentage of dead cells in control tubes which which contained NHGG and complement. Cytotoxic indices for each ATGG preparation were calculated as the dilution of a 10 mg/ml preparation which produced 50% cytotoxicity (cell death) of normal thymocyte target cells.

ATGG administration. ATGG was injected into the marginal ear vein as 30 mg doses in approximately 2 ml volumes.

Adsorption of ATGG with thymocytes. Thymus cells were harvested as previously described and divided into aliquots which gave a packed cell volume of 0.4 ml in each of

four 15 ml conical centrifuge tubes. Approximately 90 mg of ATGG contained in 6 ml was mixed with one aliquot of thymus cells. This mixture was incubated with occasional shaking for 30 minutes at 37° C in a water bath. The suspension was then centrifuged at 1200 rpm for 10 minutes. The supernatant fluid (ATGG) was then mixed with a second aliquot of T-cells and treated as above. This procedure was repeated two more times and then a cytotoxicity index was determined on the adsorbed ATGG in order to determine the efficiency of the adsorption procedure.

Immunization schedule for antigenic competition studies. One ml of a 20% suspension of HRBC was injected iv followed 3 days later with an iv injection of one ml of a 10% suspension of SRBC. This regimen of antigen administration was previously shown to produce an optimal HRBC induced suppression of the immunological response to SRBC in the rabbit (Thorne, 1972).

Experimental Protocol

Experimental group I. This experiment was designed to determine the schedule of ATGG administration which would best exhibit optimal suppression of the anti-SRBC response. The 1X group of animals received a single injection of 30 mg ATGG two days prior to SRBC; the 2X group received two 30 mg injections of ATGG, two days prior to and on the same

day as SRBC challenge, and the 3X group received three injections of ATGG, two days before, the same day and two days after SRBC immunization. Serum samples were taken from each animal and serologically assayed for SRBC hemolysins.

Experimental group II. This experiment was designed to determine the effect of 3xATGG treatment prior to SRBC immunization. Rabbits received three iv injections of 30 mg of ATGG at two day intervals followed one day later with an iv injection of one ml of a 10% SRBC suspension. The humoral immune response to SRBC was determined by both splenic anti-SRBC PFC on the 5th day after immunization and the serum hemolysin response.

Experimental group III. This experiment was designed to determine the effect of HRBC priming on the humoral antibody response to SRBC. These animals were treated as mentioned earlier (Immunization schedule for antigenic competition studies.). Anti-SRBC responsiveness was determined by the day 5 PFC assay and the serum hemolysin titers.

Experimental group IV. The experimental protocol for treatment of this group of animals is presented in Figure 3 . In order to determine the effect of ATGG treatment on the HRBC-induced suppression of the anti-SRBC response, rabbits received three iv injections of 30 mg of ATGG on days (-5), (-3) and (-1) with respect to SRBC immunization

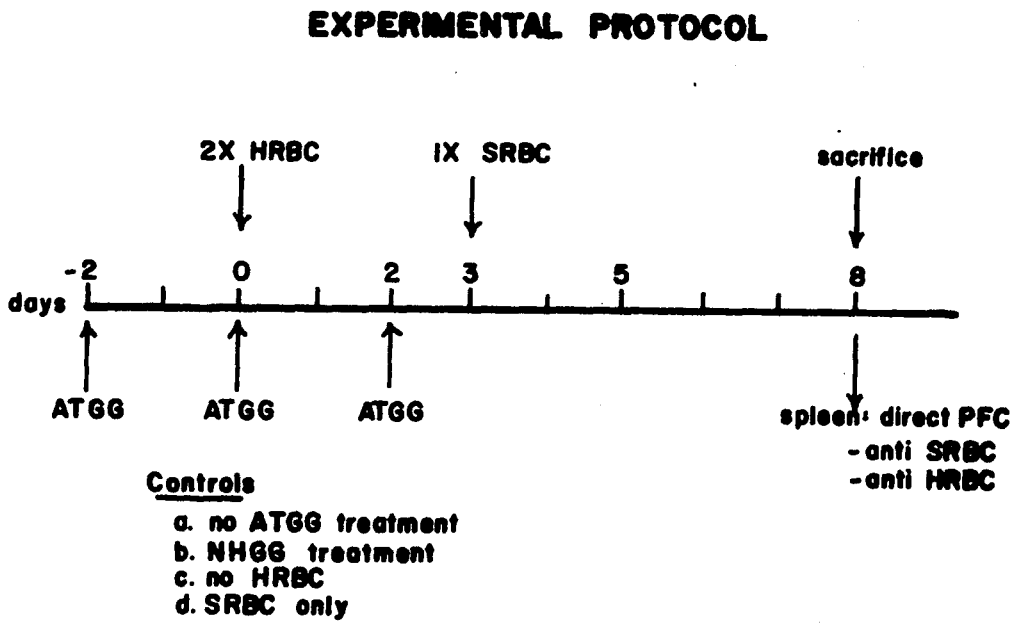

Figure 3. The experimental design for the study of the effects of ATGG-treatment on the HRBC-induced suppression of the anti-SRBC response.

(day 0). The animals received one ml of 20% HRBC on day (-3) followed on day 0 with one ml of 10% SRBC. The effect of ATGG was controlled by substituting either ATGG which had been adsorbed with thymocytes (Adsorption of ATGG with thymocytes.) or NHGG for the ATGG in the above schedule. The immune responsiveness for this experimental group and the controls was determined by both the day 5 splenic PFC assay and serum anti-SRBC hemolysin activity.

Analysis of Data

Statistical comparisons of pertinent groups were accomplished using the Student's t test. All p values, means and standard deviations from the means were obtained with the use of a Monroe Model 1766 calculator (Litton Industries, Orange, New Jersey) programmed with the 3011-S program.

Chapter 3

RESULTS AND DISCUSSION

Reconstitution Studies

The first aspect of this study was directed toward the elucidation of the nature of the helper function of the T-cell during the initiation of the humoral response to SRBC in the rabbit.

Control group. The normal humoral antibody response to a single iv injection of SRBC in 8 to 10 week old rabbits was monitored by two immunoassay methods. One group of 13 animals was immunized with 1.0 ml of a 10% SRBC suspension and seven animals were sacrificed on day five and six animals on day seven after immunization and their spleens were tested for direct PFC numbers. Tables 6 and 7 present the individual day five and day seven anti-SRBC PFC responses per million nucleated spleen cells and per whole spleen for each animal. The mean number of day seven PFC per whole spleen was 32,376± 20,166 and per million cells was 51± 14.4 (Table 7). These PFC values served to represent the "normal" response against which the splenic PFC levels of thymectomized, x-irradiated experimental groups were compared and evaluated.

Table 6.--Individual splenic plaque-forming cell responses of 8-10 week old rabbits on day 5 after immunization with 1 ml of a 10% SRBC suspension

Animal No.	PFC per 10^6 spleen cells		PFC per whole spleen	
	mean	range	mean	range
A	66	59-73	45,990	41,000-51,030
19	82	76-91	43,000	41,000-47,500
24	54	24-78	22,680	9,996-32,800
24H4	62	51-86	45,020	36,226-68,400
36	87	72-93	58,800	48,800-66,400
37	86	68-96	41,100	31,800-48,600
38	70	61-74	55,000	49,000-62,000
mean (±S.D.)	72.4 (±12.8)		40,214 (16,058)	

Table 7.--Individual splenic plaque-forming cell responses of 8-10 week old rabbits 7 days after iv antigenic challenge with 1 ml of 10% SRBC

Animal No.	PFC per 10^6 spleen cells		PFC per whole spleen	
	mean	range	mean	range
455	48	36-54	22,080	16,400-24,800
454	33	25-45	33,660	25,800-46,200
466	40	22-56	15,603	8,618-22,024
471	52	45-60	68,640	59,400-78,600
49	58	50-68	15,424	13,200-17,800
41	74	71-83	38,850	35,500-41,500
mean ($\pm$S.D.)	50.8 ($\pm$14.4)		32,376 ($\pm$20,166)	

The mean PFC numbers on day five were 72± 12.8 per 10^6 spleen cells and 40,214± 16,058 per whole spleen. Day five was chosen as the time at which to assay spleens for PFC activity for the remaining experiments since the maximum effects of HRBC-induced suppression are seen at this time.

The SRBC hemolysin activities served as the second response parameter. Figure 4 presents the mean $\log_2$ anti-SRBC hemolysin titers for prebleed and post immunization serum samples collected on days two, four, five, seven, 10, 15, 20, 25 and 30. Sheep red blood cell hemolysin titers for serum samples collected through seven days from the six animals sacrificed for PFC data and through day 30 from six additional animals were determined. This response curve served as the normal response and was used to compare the hemolysin responses observed in x-irradiation, competition and ATGG-treated experimental groups.

Experimental groups T-X-I and T-X-BM-I. Landry (1974) had previously shown that rabbits which received a midline x-ray dose of 900 R did not respond to SRBC immunization even when reconstituted with 20 x 10^8 allogeneic nucleated BM cells. One animal out of 12 in that study did show a significantly delayed hemolysin response which began on or about the 14th day following immunization and reconstitution. It was speculated that this delayed response

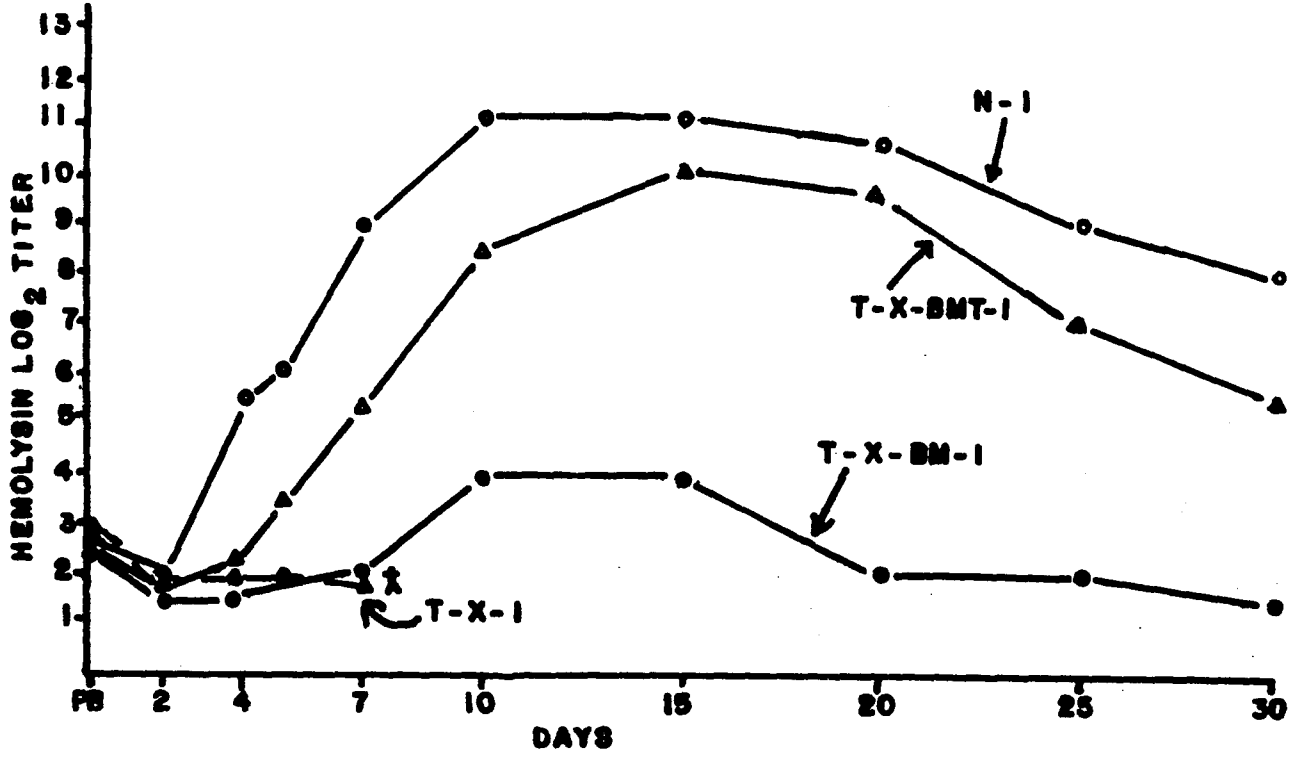

Figure 4. Anti-SRBC hemolysin humoral antibody responses for eight to ten week old rabbits immunized with 1xSRBC (N-I); thymectomized, x-irradiated (900R) and immunized (T-X-I); thymectomized, x-irradiated, BM reconstituted and immunized (T-X-BM-I), or thymectomized, x-irradiated, BM and thymus (cortisone-treated) reconstituted and immunized (T-X-BMT-I). Each response curve represents the mean $\log_2$ hemolysin titers for all rabbits tested.

was the result of the differentiation of lymphoid stem cells present in the BM to T-cells under the influence of the radiation resistant epithelial structure in the thymus. It is also possible that the thymus tissue may harbor radioresistant T-cells which are able to regenerate functional helper cells if the thymus is not removed from the experimental animal (Kadish and Basch, 1975). It thus became necessary to surgically remove the thymus lobes prior to x-irradiation in order to avoid and/or eliminate a potential T-cell source.

Experiments were designed to determine if thymectomy had any influence on SRBC responsiveness in x-irradiated rabbits which were either non-reconstituted (T-X-I) or reconstituted with $20x10^8$ nucleated littermate BM cells (T-X-BM-I). Table 8 shows the individual anti-SRBC PFC responses per million spleen cells and per whole spleen for thymectomized, x-irradiated (900R) immunized with 1.0 ml of a 10% SRBC suspension after reconstitution with BM cells or non-reconstituted. The T-X-I group had a mean PFC response of 2.2 ± 2.6 per 10^6 nucleated cells and 60 ± 48 per whole spleen on the 5th day post immunization and the T-X-BM-I showed a mean PFC response of 3.0 ± 1.8 per 10^6 cells and 1924 ± 2797 per whole spleen. The splenic PFC numbers for each of these experimental groups does not exceed background splenic PFC activity (7.4 per 10^6 and 5,844 per whole

Table 8.--Individual anti-SRBC PFC responses of 8 to 10 week old, adult thymectomized, $x_g$irradiated (900R) rabbits either reconstituted by using 20 x 10^8 nucleated BM cells (T-X-BM-I) or non-reconstituted (T-X-I). All animals were immunized with 1.0 ml of a 10% SRBC suspension

Animal No.	Exp. Grp.	PFC per 10^6 spleen cells		PFC per whole spleen	
		mean	range	mean	range
87	T-X-BM-I	1.0	0-4	356	0-1424
88	T-X-BM-I	2.1	0-8	363	0-1440
92	T-X-BM-I	5.2	2-9	6,104	2,400-10,800
57	T-X-BM-I	3.6	0-12	875	0-2,400
mean (± S.D.)		3.0 (± 1.8)		1,924 (± 2,797)	
89	T-X-I	0.3	0-1	10	0-33
51	T-X-I	0.5	0-2	35	0-140
82	T-X-I	6.0	3-9	121	60-180
53	T-X-I	2.1	0-7	73	0-245
mean (± S.D.)		2.2 (± 2.6)		60 (± 48)	

spleen) for non-immunized rabbits (Landry, 1974).

Figure 4 presents the $\log_2$ anti-SRBC hemolysin response curves for these two experimental groups compared to the control group. The hemolysin responses reflected the PFC numbers in that neither group produced significant anti-SRBC hemolysin antibody. One animal, out of seven, in the T-X-BM-I group showed a delayed low hemolysin antibody response, which could have been due either to the presence of residual epithelial tissue left from incomplete thymectomy or to the infusion of a significant population of mature T-cells which may have migrated to the donor bone marrow (Ozer and Waksman, 1972). It is also possible that the response to SRBC is not totally a T-dependent response and this represents a partially T-independent response which is delayed and less than optimal (Miller, 1975).

Although BM infusion did not restore immunological responsiveness to thymectomized, x-irradiated animals, it did enhance their viability. None of the non-reconstituted animals lived beyond the 7th day after x-irradiation, whereas three out of four of the T-X-BM-I animals which were not sacrificed for PFC assay lived through the 18th day, two beyond day 30 and one through day 75. BM infusion also restored the peripheral WBC levels to near normal while counts in non-reconstituted animals remained severely suppressed (Figure 5). Thymectomy prior to x-irradiation and BM recon-

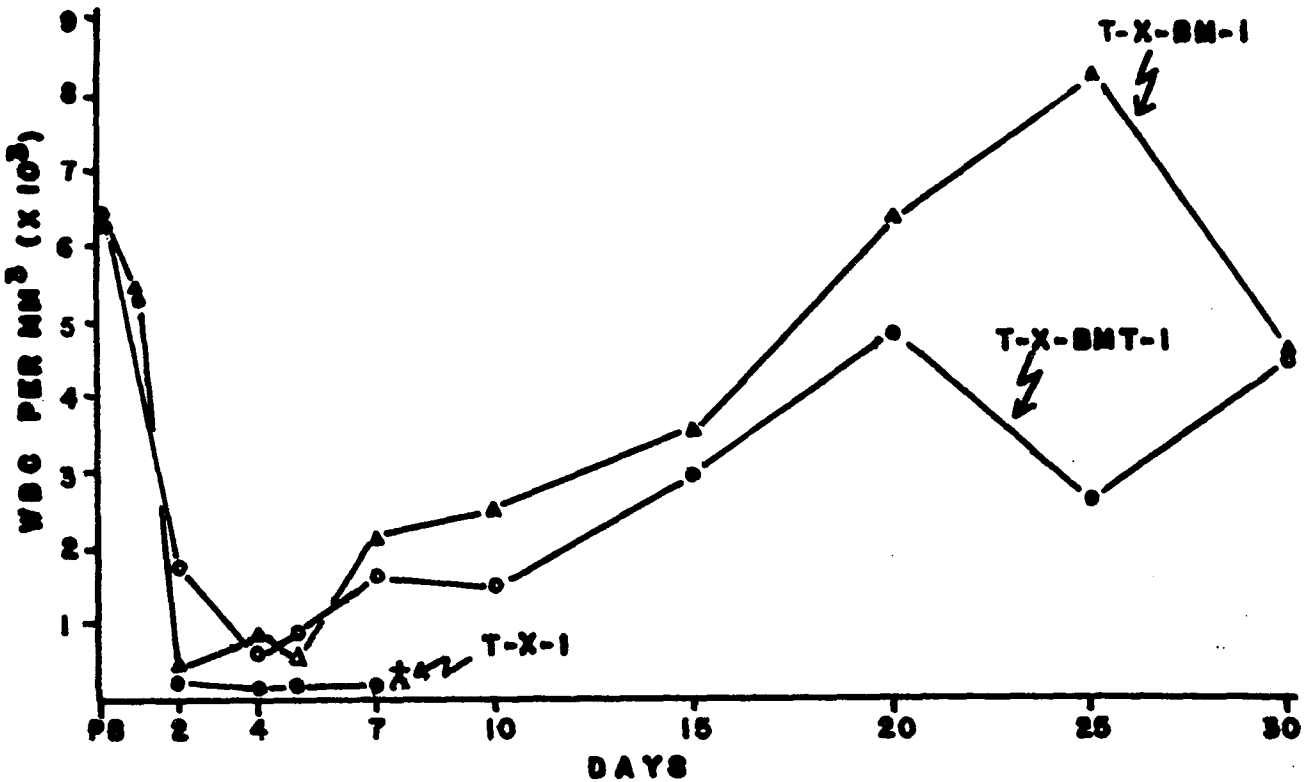

Figure 5. White blood cell counts per cu. mm. versus time for eight to ten week old rabbits thymectomized, x-irradiated; non-reconstituted (T-X-I), reconstituted with 20 x 10^6 nucleated bone marrow cells (T-X-BM-I) or reconstituted with the total BM and thymus cell populations from cortisone treated donors (T-X-BMT-I).

stitution appeared to increase the receptiveness for allogeneic bone marrow cells since these animals lived longer and maintained higher WBC counts than did non-thymectomized animals treated in the same manner (Landry, 1974). Thymectomy most probably leads to the elimination of the source of radioresistant T-cells in the host which are responsible for cell mediated immunity and which could ultimately reject allogeneic BM cell transplants. This is supported by evidence in the mouse that there is thymic regeneration in lethally x-irradiated animals which is independent of bone marrow or other stem cells (Kadish and Basch, 1975). This suggests that there are mature thymus cells within the thymus structure which are relatively radioresistant and are capable of regenerating the corticomedullary lymphocyte population. Thymectomy with x-irradiation possibly eliminate cells of host origin capable of graft rejection.

TX-BMT-I Group. Since BM alone did not successfully reconstitute immunological responsiveness, most probably because of the absence of a T-cell population, attempts were made at reconstituting thymectomized, x-irradiated rabbits with both BM and thymus cell populations. The addition of DNase to TCM 199 (used for T-cell harvesting) made thymus cell infusions possible. T-cell infusions in combination with BM from non-treated donors were only sporadically successful in restoring immunological responsiveness. This

marginal effectiveness could possibly be attributed to mature immunocompetent T-cells (10%) of the thymus with a relatively large number of immature cortical T-cells (90%). A more competent T-cell population was obtained by injecting the donors with hydrocortisone acetate 36 hours prior to collection of BM and T-cell populations. This treatment lowered the yield of donor thymocytes to less than 10% of what had been previously collected from non- hydrocortisone treated animals. This treatment also reduced the number of BM cells collected, in most cases, by more than 50%. It was, therefore, necessary to infuse each recipient with the total BM and thymus cell yields from the selected treated donor litter mate. The number of BM cells infused ranged from 5×10^8 to 19×10^8 and the number of thymocytes ranged from 9×10^7 to 13×10^8.

Table 9 presents the anti-SRBC PFC responses in the spleens of BM and T-cell reconstituted recipients. There seems to be a need for a critical number of reconstituting T-cells as the two animals which received less than 2×10^8 cells (animals number 37 and 39) had PFC responses which were less than background; whereas, those animals which received at least this number showed a normal PFC response per 10^6. The mean number of PFC for animals number 33, 31, 43 and 47 per 10^6 spleen cells was 48 ± 8.2 and $12{,}416 \pm 6{,}955$ per whole spleen. BM cells and thymocytes, when given in sufficient numbers from cortisone-treated littermate donors,

Table 9.--Individual splenic anti-SRBC PFC responses in adult thymectomized, x-irradiated (900R), reconstituted with the total population of BM and thymus cells from cortisone-treated littermate donors immunized with 1.0 ml of a 10% SRBC suspension

Animal No.	Cell pop. $\times 10^8$		PFC per 10^6 spleen cells		PFC per whole spleen	
	Thymus	BM	mean	range	mean	range
33	2.6	9.1	40	27-52	4,800	3,240-6,240
31	12.7	18.8	43	34-48	17,350	15,300-21,600
43	5.0	12.0	58	50-70	8,352	7,200-10,080
47	12.0	5.0	52	45-60	17,160	14,850-19,800
37	0.9	9.0	7	0-14	581	0-664
39	1.0	5.2	6	0-16	780	0-1,300
Normal (N-I)*			50.8 ($\pm$ 14.4)		32,376 ($\pm$ 20,166)	

*from Table 7

did synergistically restore immunological responsiveness to SRBC in thymectomized lethally irradiated rabbits. The addition of mature thymocytes to the reconstituting cell population significantly increased the PFC responsiveness of thymectomized lethally irradiated rabbits as statistical analysis yielded a $p < 0.001$ when comparing the PFC per 10^6 of the four responding animals of the T-X-BMT-I group to both T-X-I and T-X-BM-I. The increase in PFC per whole spleen for T-X-BMT-I was also statistically significant as $p < 0.001$ compared to T-X-I and $p < 0.05$ compared to T-X-BM-I. The percentage of cells in the spleens of T-X-BMT-I (recipients number 31, 33, 43 and 47) committed to anti-SRBC antibody production was not significantly different from that of the control group cells, as $p > 0.20$ when comparing PFC/ 10^6 of T-X-BMT-I with the 10^6 response of the control group. There were, however, significantly fewer total anti-SRBC producing cells in the T-X-BMT-I spleens than in the control group spleens, $p < 0.10$.

Figure 4 shows the mean $\log_2$ anti-SRBC response curve of the T-X-BMT-I group compared to that of the N-I, T-X-BM-I and T-X-I groups. The hemolysin response curve for T-X-BMT-I rabbits approached that of the control group, N-I. It was,however,slightly depressed and exhibited a longer lag period. The hemolysin titers in the T-X-BMT-I animals reflected the splenic PFC responses with antibody

Table 10.--Summary of the mean day 7 splenic anti-SRBC PFC responses for normal (N-I); thymectomized, x-irradiated (T-X-I); thymectomized, x-irradiated, bone marrow reconstituted (T-X-BM-I); thymectomized, x-irradiated, bone marrow and thymus reconstituted (T-X-BMT-I) experimental groups

Experimental group	Mean PFC responses			
	per 10^6	p values[b]	per whole spleen	p values[b]
N-I	50.8 ($\pm$ 14.4)	—	32,376 ($\pm$ 20,166)	—
T-X-I	2.2 ($\pm$ 2.6)	<0.001	60 ($\pm$ 48)	<0.001
T-X-BM-I	3.0 ($\pm$ 1.8)	<0.001	1,924 ($\pm$ 2,797)	<0.001
T-X-BMT-I[a]	48.3 ($\pm$ 8.3)	>0.2	12,416 ($\pm$ 6,955)	<0.10

[a] represensts only those animals which received more than 2×10^8 thymus cells (animals number 33, 31, 43, and 47)

[b] p values represent a comparison of each group to the N-I group

levels significantly greater than in animals reconstituted with BM alone.

These findings are in agreement with the results reported by Ozer and Waksman (1972) that there exists a requirement for more than one lymphoid cell population to cooperate and mount an immune response to SRBC in the rabbit. These observations also agree with the two-cell concept which has been reported for the mouse (Claman et al.,1966; Miller and Mitchell, 1968) and the rat (Baum et al., 1969) in that T and B-cells must cooperate in order for antibody synthesis to occur (T-dependent antigens). It would appear that cortisone treatment destroys only the immature cortical thymocyte population in the rabbit, since T-cell helper function is present in cortisone treated animals. The functional B-cell (pre-AFC) in rabbit bone marrow apparently is relatively insensitive to cortisone treatment, since AFC activity can be restored with infusion of cortisone treated bone marrow.

The reconstitution studies reported here with cortisone treated cells <u>in vivo</u> confirm work done <u>in vitro</u> with cortisone treated mouse thymocytes and T-cell depleted spleen cells (Schimpl and Wecker, 1971). These investigators reported that they were able to achieve a primary antibody response <u>in vitro</u> to SRBC with cortisone treated thymocytes and anti-θ serum treated spleen cells. Previous investi-

gators were unable to use thymocytes for a T-cell source in the primary in vitro response due to the large number of immature cells. It was necessary to use a more mature population of T-cells (eg. thoracic duct cells). Schimpl and Wecker (1971) showed that cortisone-treatment selected for an equivalent, mature population in the thymus.

Low dose irradiation experiments. Experiments were designed to determine the relative radioresistance of thymus-derived lymphocytes. Rabbits received a midline dose of 600R and then were either reconstituted with $20x10^8$ littermate BM cells or served as non-reconstituted control animals. Both groups were prebled, immunized with 1.0 ml of a 10% SRBC suspension and serum samples were taken two, four, five and seven days post immunization at which time the animals were sacrificed and splenic PFC assays were performed.

Figure 6 presents the $\log_2$ hemolysin responses determined for both reconstituted and non-reconstituted animals. A midline irradiation dose of 600R effectivly eliminated the capacity of these animals to respond to a SRBC challenge, since the anti-SRBC hemolysin response in non-reconstituted animals never exceeded background antibody levels. BM cell infusion restored partial immunological responsiveness since there was a significant rise in ati-SRBC titer in BM reconstituted rabbits. The response achieved in the reconsti-

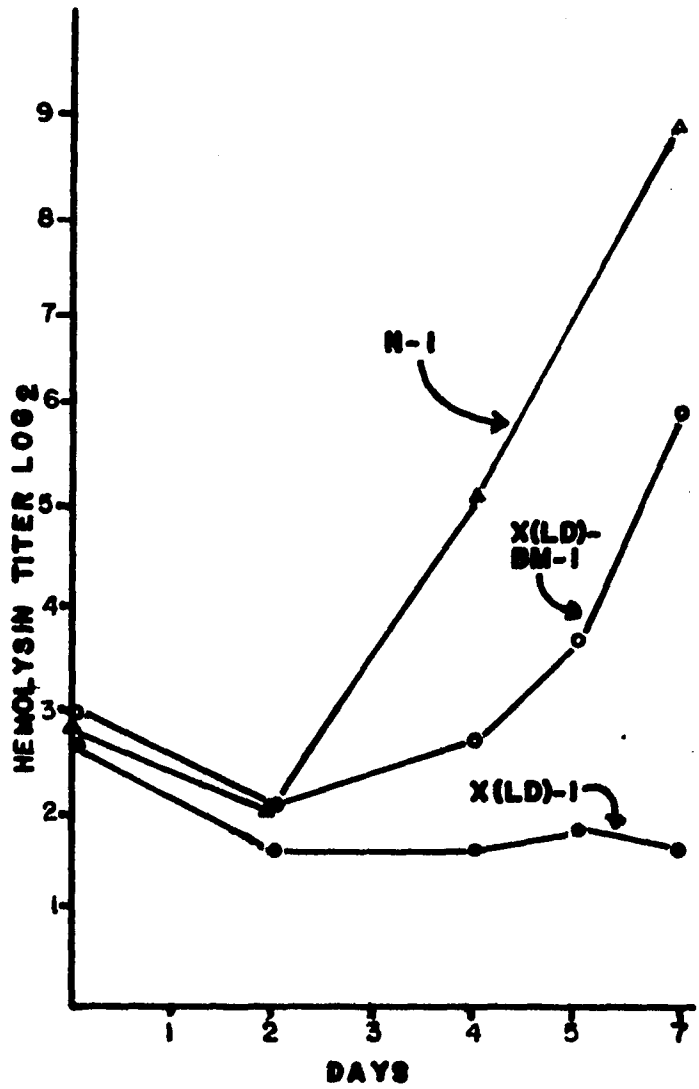

Figure 6. Mean $\log_2$ anti-SRBC hemolysin titers versus time (7 day period) for normal rabbits immunized with SRBC (N-I), 600R x-irradiated and SRBC immunized (X(LD)-I) and 600R x-irradiated, reconstituted with 20 x 10^8 nucleated BM cells and SRBC immunized (X(LD)-BM-I).

tuted animals was less than that is seen in normal animals. This is as would be expected since the T-cells would be of host origin and the B-cells would be from the donor and optimal cooperation might not be expected, since the cooperation between allogeneic T and B-cells generally does not compare to syngeneic cooperation.

The PFC assays in this experiment failed to yield any detectable plaques, probably due to some technical error in the assay procedure, rather than to an actual absence of PFC since the hemolysin responses observed in these animals would indicate some PFC activity.

Unfortunately, immediately following this experiment the rabbit colony became infected with Yersinia pseudotuberculosis. When "infected" animals are subjected to stress, such as irradiation, they rapidly succumb to the infection. This prevented repeating the PFC data at that time but led to a different experimental approach and the second aspect of this study. As a consequence, the low dose experiments were not pursued further.

The low dose experiment suggested that the T-cell was resistant to an irradiation dose of 600R and that the B-cell function was sensitive. With this in mind, the next series of experiments was designed to eliminate immunocompetent B-cells with low dose irradiation and the T-cells by thymectomy and in vivo anti-thymocyte antibody treatment.

It was the preliminary studies with respect to the use of anti-thymocyte gamma globulin as a tool for eliminating the T-cell helper function that led to the second aspect of this study.

Suppressor Function

Homogeneity of horse IgG. Figure 7 shows the immunoelectrophoresis pattern for rabbit anti-whole horse serum which was allowed to diffuse and react with electrophoresed whole serum and the DEAE chromatography IgG fraction. Immunoelectrophoresis of the DEAE fraction resulted in a single line of precipitation which had migrated toward the cathode, as did the IgG component of the HS, with no detectable contaminating components present.

Cytotoxicity indices. Each ATGG fraction prior to use was adjusted to give a concentration of 10 mg protein per ml. The dilution of the various test bleed samples taken from Horse 1 and 2 which gave a 50% CI when tested against normal thymus cells was determined. Two horses were immunized as described in the Materials and Methods, Horse 1 in the summer of 1973 and Horse 2 in the summer of 1974. It was found that the three week serum sample (post immunization) for both horses yielded ATGG with the highest cytotoxicity index. The three week serum sample for Horse 1 showed a 50% CI at a 1/40 dilution; whereas, the three week sample

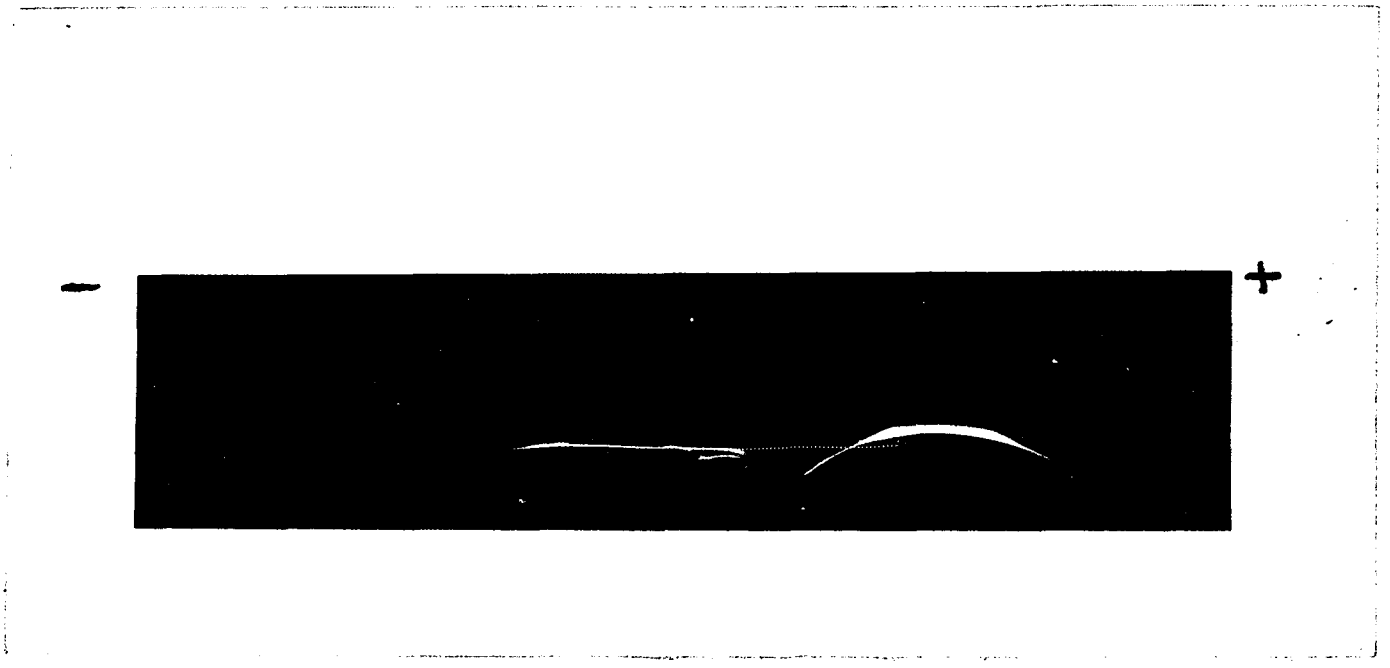

Figure 7. Immunoelectrophoresis pattern for horse serum and for the DEAE-chromatography fraction (ATGG) versus rabbit anti-horse serum.

for Horse 2 showed a 50% CI at a 1/32 dilution. The ATGG fraction from Horse 1 proved to have the greater effect *in vivo* and was used in most experiments unless otherwise specified. The Horse 1 serum was used in prliminary *in vitro* studies and the supply was exhausted before completion of the *in vivo* experiments. It, therefore, became necessary to use Horse 2 serum ATGG in later experiments.

Optimal schedule for ATGG administration. The following experiment was designed to determine the schedule for ATGG administration which was optimal for suppression of immunological responsiveness to SRBC. As stated above it was used for the intention of depleting T-cell helper function which is necessary for optimal immunological responsiveness to SRBC. Figure 8 presents the mean $\log_2$ hemolysin responses for animals which received one iv injection of 30 mg of ATGG two days prior to SRBC immunization (1X); animals which received two ATGG injections (2X) one, two days prior and one, on the same day as SRBC immunization, and the 3X group which received a third injection of ATGG two days following immunization. Unexpectedly, all three ATGG treated groups showed an early response which also was elevated when compared to control N-I group animals through seven days. The apparent ATGG-enhancing effect was apparently related to the number of ATGG injections administered. The 3X group showed the greatest degree of hemolysin enhance-

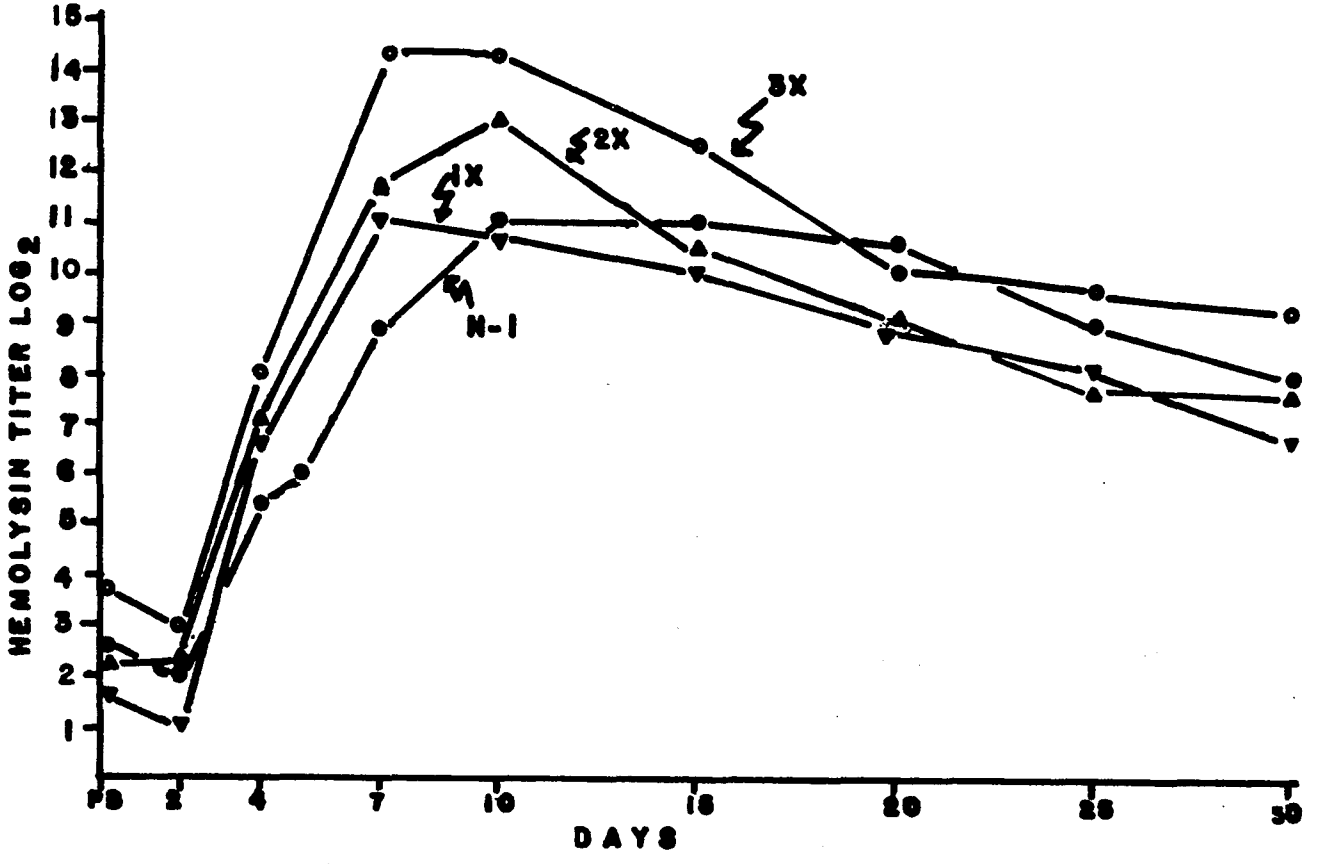

Figure 8. Mean $\log_2$ hemolysin response curves for normal rabbits immunized with 1xSRBC (N-I) or for rabbits treated with 30 mg of ATGG: two days prior to 1xSRBC immunization (1X), two days prior to and on the same day as 1xSRBC immunization (2X), and two days prior to, on the same day and two days post 1xSRBC immunization (3X).

ment and antibody levels remained elevated for a longer period of time than the 1X or 2X groups. These findings suggested at least two possible means in which ATGG may be functioning. Either the ATGG was selectively eliminating some suppressor function responsible for homeostasis of normal humoral responsiveness or it was acting in some way to enhance antibody formation. It followed that if a suppressor function were being eliminated or neutralized then ATGG might act similarly to alleviate antigen-induced suppression which has been observed in antigenic competition.

Antigen-induced suppression (AIS). Thorne (1972) had previously established a competition system in rabbits utilizing heterologous erythrocyte antigens. She found that 1.0 ml of a 20% HRBC suspension (2X) injected iv optimally suppressed the response to a 1X (1.0 ml of a 10% suspension) SRBC suspension administered three days later. This schedule and erythrocyte immunization dose was used in the following experiment which utilized eight to ten week old rabbits. The SRBC hemolysin response of one experimental group was followed through 30 days. A second group of six animals was sacrificed on the fifth day following SRBC immunization and splenic anti-SRBC and anti-HRBC PFC assays were done. The mean $\log_2$ anti- SRBC hemolysin responses for the AIS group are shown in Figure 11 along with

hemolysin responses for the N-I group rabbits. The administration of a priming dose of HRBC markedly suppressed the hemolysin response to a subsequent SRBC challenge, which remained depressed through 30 days.

Table 11 presents the splenic anti-SRBC and anti-HRBC PFC responses for the AIS group on the fifth day after SRBC injection. These animals had a mean anti-SRBC PFC response of 29± 9.3 per million nucleated cells and 24,342± 16,728 per whole spleen. These values are significantly depressed ($p<0.001$ for both groups) when compared to day five responses for N-I group rabbits which show 72± 12.8 PFC/10^6 and 40,214± 16,058 PFC/whole spleen (Table 6). The mean anti-HRBC PFC numbers for the AIS group were 19± 5.2 per 10^6 cells and 15,167± 6,280 per whole spleen.

ATGG treated AIS group. Table 12 presents the anti-SRBC and anti-HRBC PFC responses for competition animals which had been treated with 30 mg of ATGG two days prior, the same day and two days following HRBC priming. In this schedule the animals received SRBC one day after the final ATGG injection. Half of the animals in this group were treated with ATGG from Horse 1 and the other half with ATGG from Horse 2. Both were effective in alleviating AIS although the PFC values obtained for the various assays were somewhat different. Horse 1 ATGG showed an earlier enhance-

Table 11.--Individual splenic plaque-forming cell responses of rabbits primed with 2xHRBC three days prior to 1xSRBC immunization (AIS) assayed on day five after SRBC immunization

Animal No.	SRBC PFC per			
	10^6 spleen cells		whole spleen	
	mean	range	mean	range
92	40	27-46	55,300	37,800-64,400
40	26	13-34	18,000	8,400-30,400
44	30	23-38	31,500	30,000-45,600
45	33	18-51	16,250	9,000-26,000
46	13	9-17	11,200	8,000-14,400
49	35	21-51	13,800	8,400-20,400
mean(±S.D.)	29(± 9.3)		24,342(± 16,728)	

Table 12.--Individual splenic PFC of rabbits primed with 2xHRBC three days prior to 1xSRBC immunization and treated with 3xATGG (30 mg) on days (-5), (-3) and (-1) in relation to SRBC immunization (ATGG-AIS)

Animal No.	SRBC PFC per			
	10^6 spleen cells		whole spleen	
	mean	range	mean	range
74[a]	119	98-131	119,000	98,000-131,000
71[a]	126	88-134	190,200	132,000-201,000
75[a]	83	80-98	140,000	108,000-176,800
82[b]	73	67-83	26,100	23,400-30,000
83[b]	84	79-98	43,200	37,600-47,200
88[b]	57	47-62	55,500	45,000-62,000
mean (±S.D.)	90 (± 26.8)		95,666 (± 64,268)	

[a]Horse 1 (1973) - three week serum sample

[b]Horse 2 (1974) - three week serum sample

ment; whereas, Horse 2 ATGG treated animals showed a more persistent enhanced response. The mean anti-SRBC PFC responses for these animals were 90± 26.8 per 10^6 spleen cells and 95,666± 64,268 per whole spleen on the fifth day post SRBC challenge. ATGG treatment appeared to eliminate or neutralize the suppression induced by HRBC administration since these values were significantly ($p<0.001$) elevated when compared to the non-ATGG treated AIS group. This group also showed a marginal increase in anti-SRBC PFC when compared to the non-competitive N-I response ($p<0.2$). There was also an elevation in the numbers of the anti-HRBC splenic PFC, since the ATGG-AIS group showed PFC values of 52± 28.0 per 10^6 and 52,283± 39,093, which represented a significant increase in anti-HRBC PFC responsiveness ($p<0.001$) when compared to the AIS group.

Figure 9 shows the mean anti-SRBC $\log_2$ serum hemolysin titers through 30 days for AIS group animals treated with the Horse 1 ATGG compared to serum hemolysin levels for N-I group rabbits. The hemolysin response was both accelerated and elevated with a peak titer of $\log_2$ 13 on day seven, which is significantly greater than the day seven antibody titer of 8.9 for the N-I group animals. The elevated hemolysin titer dropped by the 10th day and was similar if not slightly depressed when compared to the N-I group through the 30th day. Figure 10 is the hemolysin response curve of

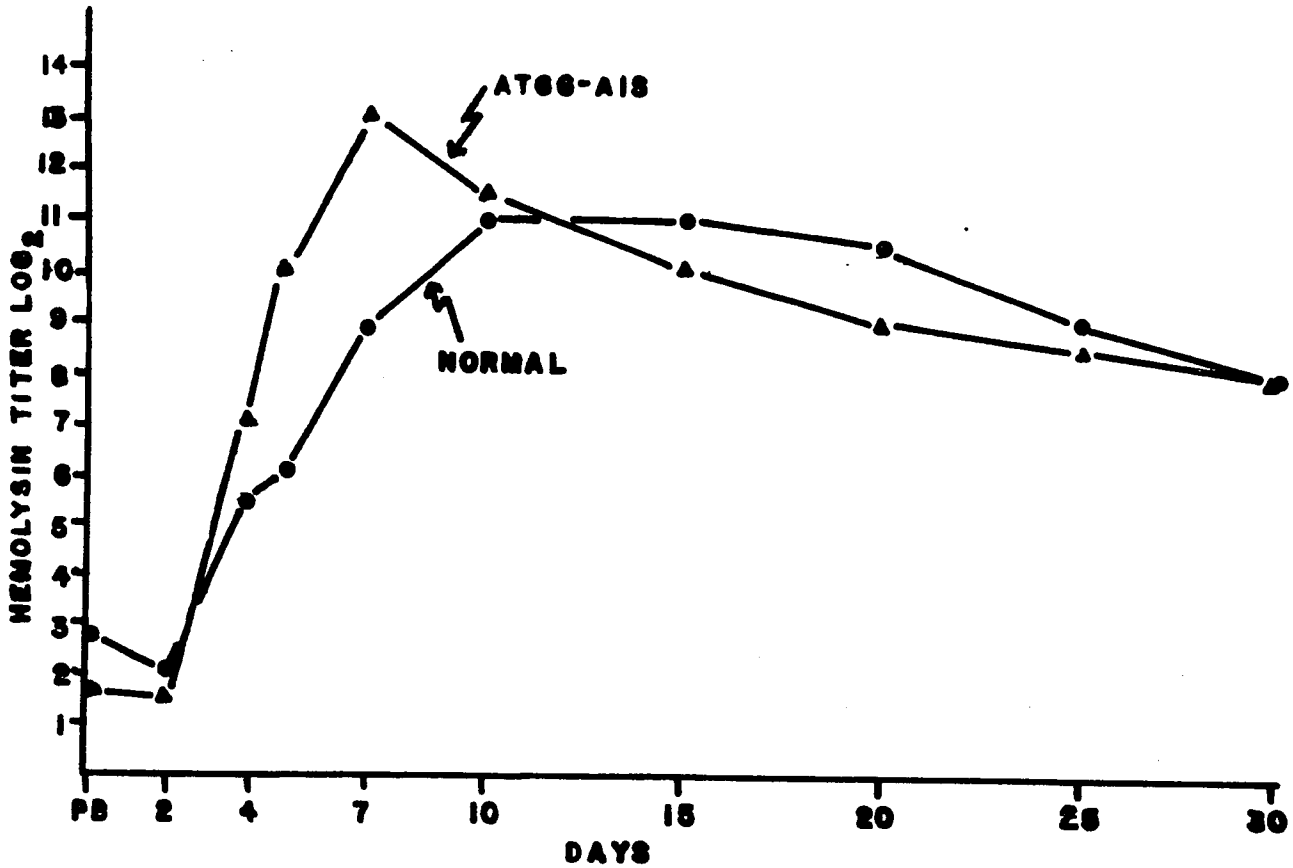

Figure 9. Mean $\log_2$ anti-SRBC hemolysin response curves for normal rabbits immunized with 1xSRBC (N-I) and for rabbits injected with 30 mg ATGG from Horse 1 on days (-5), (-3) and (-1), immunized with 2xHRBC on day (-3) and with 1xSRBC on day (0) (ATG-AIS).

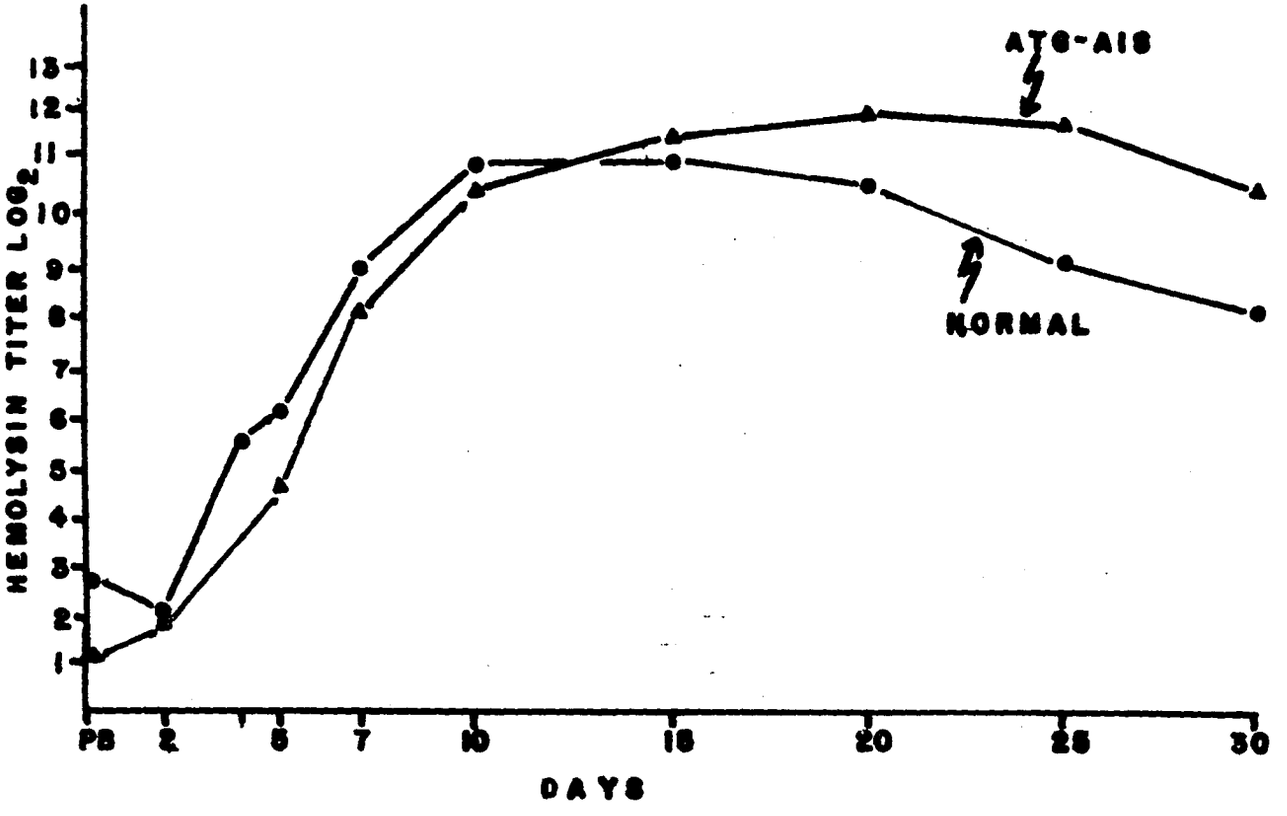

Figure 10. Mean $\log_2$ anti-SRBC hemolysin response curves for normal rabbits immunized with 1xSRBC (N-I) or injected with 30 mg ATGG from Horse 2 on days (-5), (-3) and (-1), immunized with 2xHRBC on day (-3) and with 1xSRBC on day (0) (ATG-AIS).

Horse 2 ATGG treated AIS animals. In contrast to the Horse 1 ATGG treated animals, there was no accelerated early response in these animals. There was, however, persistent hemolysin production which remained elevated through day 30. Figure 11 compares the combined mean hemolysin response curve for both these groups (ATGG from Horse 1 and 2) with that of the non-ATGG treated AIS group and the non-competitive N-I group. ATGG treatment eliminated the suppressive effects which HRBC priming injections provoked, since subsequent serum hemolysin response to SRBC for ATGG-treated AIS group animals paralleled that of the control N-I group.

ATGG treatment followed by SRBC immunization. Figure 12 presents the mean $\log_2$ hemolysin titers for rabbits which received three injections of ATGG followed one day later by SRBC immunization. There was marked anti- SRBC hemolysin activity by the seventh day and the antibody titer remained elevated through 30 days. Splenic PFC enhancement was also seen for animals in this group and showed a mean PFC response of 120 ± 48.7 per 10^6 cells ($p < 0.05$ when compared to N-I) and $79,200 \pm 49232$ ($p < 0.10$ when compared to N-I) per whole spleen.

NHGG-AIS experiment. Table 13 presents the PFC data for AIS animals which had been treated with three injections of 30 mg NHGG. The spleens of six experimental animals had mean anti-SRBC PFC numbers of 32 ± 26.0 per 10^6

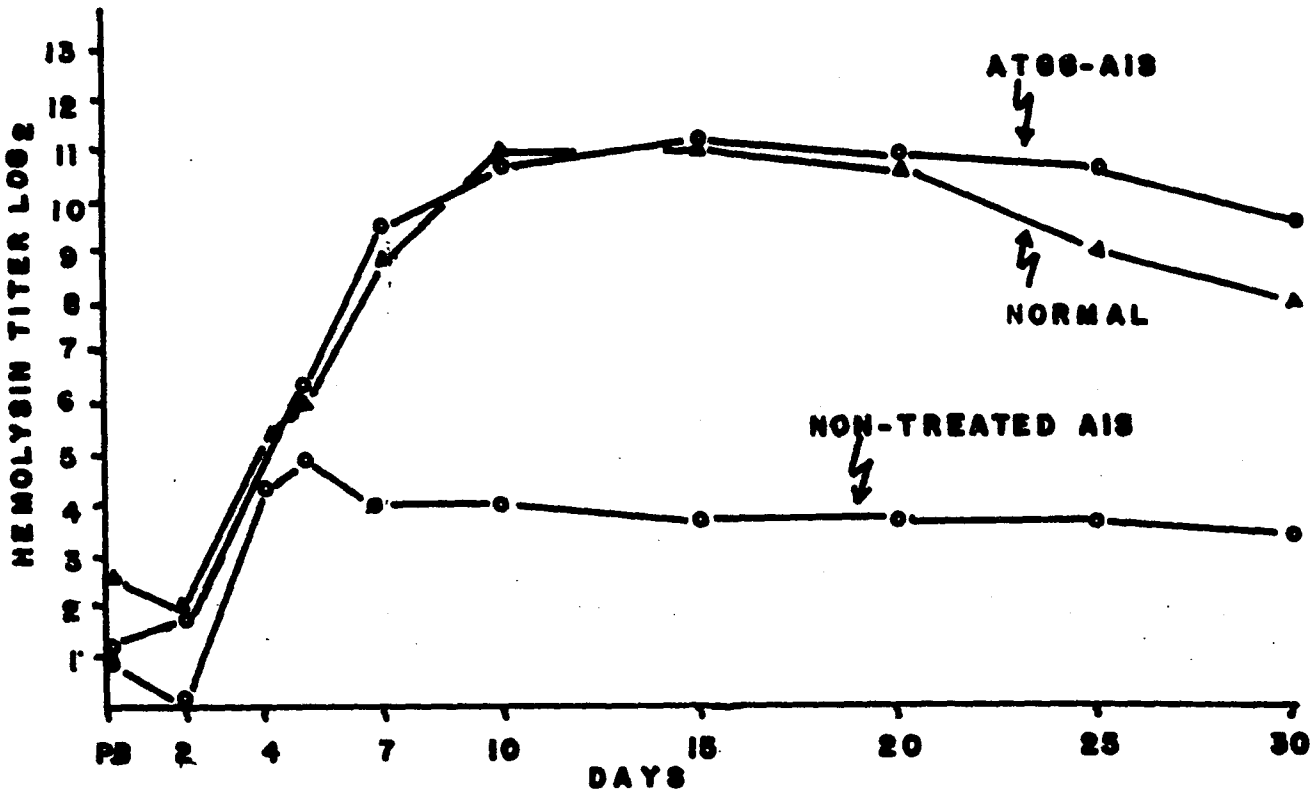

Figure 11. Mean $\log_2$ anti-SRBC hemolysin response curves for normal rabbits immunized with 1xSRBC (N-I), primed with 2xHRBC three days prior to challenge with 1x SRBC (AIS) or injected with 30 mg ATGG from either Horse 1 or 2 on days (-5), (-3) and (-1), primed with 2xHRBC on day (-3) and challenged with 1xSRBC on day (0) (ATG-AIS).

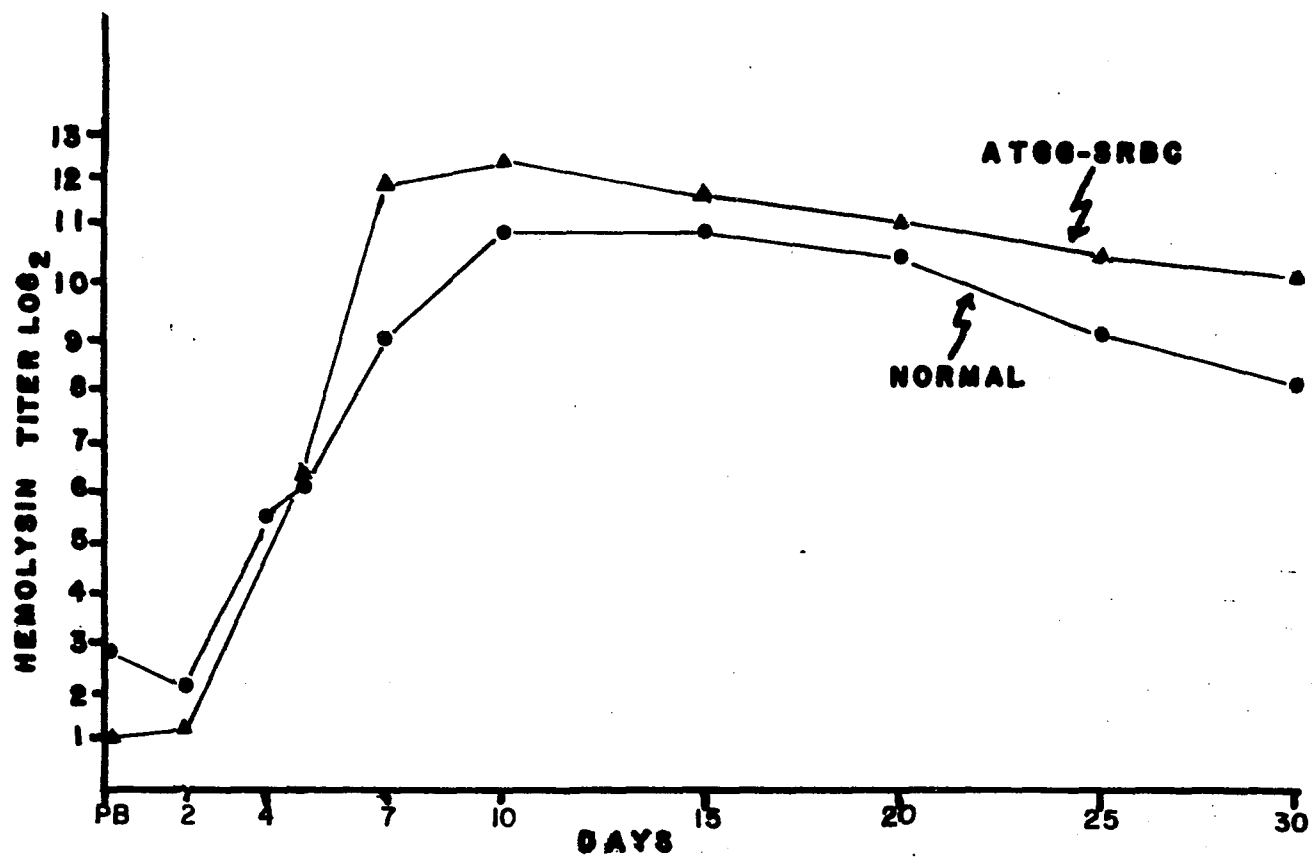

Figure 12. Mean $\log_2$ anti-SRBC hemolysin response curves for normal rabbits immunized with 1xSRBC (N-I) or treated with 30 mg ATGG on days (-5), (-3) and (-1) followed by 1xSRBC immunization on day (0).

Table 13.--Individual anti-SRBC PFC responses of animals immunized with 1xSRBC on day 0 after treatment with 30 mg ATGG on days (-5), (-3) and (-1)

Animal No.	PFC per 10^6 cells		PFC per whole spleen	
	mean	range	mean	range
251	184	163-202	124,800	111,200-137,600
265	96	87-106	45,900	42,000-51,000
270	120	102-145	31,200	26,400-37,600
252	78	68-84	31,200	27,200-33,600
253	174	126-190	141,300	108,000-171,000
254	70	66-78	100,800	95,400-111,600
mean (±S.D.)	120(±48.7)		79,200 (± 49,232)	

Table 14.--Individual splenic anti-SRBC PFC of rabbits treated with normal horse gamma globulin (NHGG) prior to the HRBC-induced suppression of the anti-SRBC response (NHGG-AIS).

Animal No.	PFC per 10^6 cells		PFC per whole spleen	
	mean	range	mean	range
54	58	40-80	66,600	45,600-92,400
53	16	8-23	21,500	17,500-24,100
56	63	52-68	53,900	46,800-61,200
53A	9	6-16	7,800	4,800-15,200
57	4	3-10	4,000	2,400-9,600
59	40	33-48	32,000	26,400-38,400
mean ($\pm$ S.D.)	32($\pm$ 26)		30,966 ($\pm$ 25,100)	

nucleated cells and 30,966± 25,100 per whole spleen. The anti-HRBC responses were 17± 19.0 PFC PER million cells and 16,275± 17,600 PFC per whole spleen. NHGG treatment provoked no significant effect on antigen induced suppression of a subsequent PFC response ($p > 0.2$) or on the anti-HRBC response ($p > 0.2$) which suggested that the active component in ATGG was not present in NHGG since the latter had little or no effect on antigen-induced suppression.

ATGG (T-adsorbed) -AIS experiment. Ninety mg aliquots of ATGG were adsorbed four successive times with 0.4 ml packed cell volumes of normal rabbit thymocytes. AIS animals received three injections of "adsorbed" ATGG as previously described two days before on the same day and two days after HRBC injection. Adsorption with thymus cells had effectively removed the anti-suppressor activity present in ATGG since splenic PFC numbers on the fifth day post SRBC injection were even more suppressed in the adsorbed ATGG treated group than in the non-ATGG-treated AIS group. The splenic PFC results are presented on Table 15 . Rabbits treated with adsorbed ATGG showed 19± 16 anti-SRBC PFC/10^6, 12,288± 12,234 anti-SRBC PFC/whole spleen, 17± 10 anti-HRBC PFC/10^6 and 9,708± 6,707 anti-HRBC PFC/whole spleen. These findings support the assumption that ATGG is selectively acting on a T-cell population responsible for suppressor activity, since specific Ab can be adsorbed from the ATGG fraction

Table 15.--Individual splenic anti-SRBC PFC responses of rabbits primed with 2xHRBC followed three days later with 1xSRBC challenge and treated with three injections of ATGG which had been adsorbed with normal thymocytes (ATGG-ads.-AIS)

Animal No.	PFC per 10^6 cells		PFC per whole spleen	
	mean	range	mean	range
1	3	1.8-3.2	1,250	900-1,600
2	4	3.2-4.0	1,480	1,280-1,600
3	46	40-51	26,800	24,000-30,600
4	17	13-23	11,400	7,800-13,800
5	30	25-32	27,900	22,500-28,800
6	15	12-18	4,900	4,100-6,300
mean ($\pm$ S.D.)	19($\pm$ 16)		12,288($\pm$ 12,234)	

Table 16.--Summary of the mean day 5 (after SRBC immunization) splenic anti-SRBC PFC responses for normal (N-I), HRBC-primed anti-SRBC response (AIS), ATGG treated AIS group (ATGG-AIS), NHGG treated AIS group (NHGG-AIS), and ATGG (thymocyte adsorbed) treated AIS group (ATGG-ads.-AIS)

Experimental group		Mean PFC responses		
		per 10^6	p values	per whole spleen
I.	N-I	72 ($\pm$ 12.8)	-	40,214 ($\pm$ 16,058)
II.	AIS	29 ($\pm$ 9.3)	<0.001	24,342 ($\pm$ 16,728)
III.	ATGG-AIS	90 ($\pm$ 26.8)	<0.2	95,666 ($\pm$ 64,268)
IV.	NHGG-AIS	31 ($\pm$ 25.6)	<0.01	30,966 ($\pm$ 25,100)
V.	ATGG-ads.-AIS	19 ($\pm$ 16)	<0.001	12,288 ($\pm$ 12,234)

p values represent a comparison of the mean PFC/10^6 of each group to that of the N-I group

with thymus cells. The first aspect of this study suggested the need for a thymus cell in addition to BM in order for lethally x-irradiated rabbist to respond with Ab synthesis. The T-cell is probably functioning in the capacity of a helper cell (antigen recognition and AFC recritment) as has been established for most other animal systems. The results presented in the latter part of this study suggest that in addition to helper function, there exists also a T-cell suppressor function which has been suggested by the investigations of others (Radovich and Talmage, 1967; Gershon and Kondo, 1971a; Okumura and Tada, 1971a,b; Armstrong et al., 1969; Baker et al., 1970a,b).

These results further suggest that the suppressor function can be assigned to a separate subpopulation of T-cells distinct from helper cells when viewed in the light of the work of others. Wu and Lance (1974) suggested that the suppressor cell belongs to a subpopulation of thymus-derived cells which are spleen-seeking and require the microenvironment of the spleen for expression. Scavulli and Dutton (1975) separated the two populations (suppressor and helper) based on their variable susceptibility to Con A stimulation and x-irradiation sensitivity. They found the suppressor cell to be susceptible to complement-mediated lysis in the presence of specific anti-thymocyte serum. Weksler et al. (1974) reported that suppressor activity was

sensitive to cortisone in contrast to cortisone-resistant helper cells. In opposition to Scavulli and Dutton and the present study they reported that suppressor activity was resistant to the action of anti-thymocyte antiserum.

Most investigators have reported that in vivo administration of ALS or ATS suppressed the humoral response to subsequent injections of SRBC and other T- dependent Ag (Lance, 1973; Baum et al.,1969; James, 1969; Kerbel and Eidinger, 1971). Others have reported that ALS treatment has little effect on the humoral response to T-dependent Ag (Baker et al., 1970; James, 1969; Stewart and Bell, 1970) and in some instances their data, if closely examined, suggest a slight enhancement in the humoral Ab response. This variability in the reported effect of anti-thymocyte antibody does suggest that there is a difference in the anti-sera preparations and that there may be antigenic as well as functional differences between helper and suppressor cell populations. It is possible that certain preparations of anti-thymocyte antisera are directed primarily toward one population of T-cells or the other, possibly dependent upon their relative concentrations in the immunizing doses or preparations. It is highly likely that there is antibody activity directed against both suppressor and helper cells. If the action of the antisera (anti-thymus) is being assayed in a T-dependent Ag system, such as SRBC, then the anti-

suppressor activity would not be apparent, since the helper activity necessary for Ab expression would also be eliminated or destroyed with the suppressor activity. This conclusion was supported by the observations of Baum et al. (1969) and Baker et al. (1970) who reported consistent enhancement of Ab synthesis in mice to specific T-independent Ag (Streptococcus pneumonia SSS III) when treated in vivo by the administration of ALS.

The enhanced response to the T-dependent Ag, SRBC, with ATGG observed in this study suggests that this antiserum preparation is directed primarily toward the suppressor function. The effect of the ATGG can be attributed to the elimination of a suppressor function rather than a stimulatory influence on T-cell helper function since horse ATGG is capable of eliminating the suppression observed in antigenic competition. Treatment with ATGG must leave helper function more or less intact in order to allow the response observed. These results suggest antigenic differences between the two subpopulations. Whether these differences reflect the presence of distinct haptenic determinants on each thymic cell subpopulation or rather, relative variations in concentrations of shared determinants can not be determined from the data at this time.

Some investigators have suggested that helper and suppressor functions are immunological expressions of the

same cell, and it is suggested that the T-cell acts initially in a helper capacity and as the immune response progresses, the T-cell switches to a suppressor function. If this were so then it would seem likely that anti- T-cell antibodies would not be effective in eliminating suppressor activity unless they were administered after the initiation of the helper activity. The fact that in this study enhancement was observed even when ATGG treatment preceded antigenic stimulation suggests that the suppressor precursor cells exist as a separate population independent of antigenic stimulation.

In conclusion, these results suggest the existence of T-suppressor cells responsible for the antigen-induced suppression observed in antigenic competition. This observed suppression may be a manifestation of a homeostatic function of the suppressor T-cell which keeps the immune response within proper limits and rather conservative, since enhancement was observed with elimination of suppressor function (especially with Horse 1 ATGG) when the animals received only a single antigen, SRBC. This regulatory function of the T-cell extends to control of responses to T-independent antigens and to cell mediated immunity as well.

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VITA

Roland Ray Arnold was born in Denver, Colorado on December 18, 1946. He received his high school diploma from Baton Rouge Senior High School, Baton Rouge, Louisiana in May, 1964. He received a Bachelor of Science degree in Microbiology from Louisiana State University in May, 1972. He served in the U. S. Army from March, 1969 to March, 1971. He has been married to the former Mona Geier since February, 1969. They have a five year old son, Todd.

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EXAMINATION AND THESIS REPORT

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Major Field: Microbiology

Title of Thesis: Helper and Suppressor Functions of Thymus-Derived Lymphocytes in the Humoral Response to Heterologous Erythrocytes in Rabbits

Approved:

R. J. Siebeling
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James R. Traynham
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Date of Examination:

July 9, 1975